Circulating Cell Free DNA Integrity Index as a Biomarker for Response to Chemotherapy in Patients with Metastatic Colorectal Carcinoma

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Abstract

Objective: Assessing plasma Cell Free DNA (cfDNA) integrity index as a biomarker for response prediction and early response evaluation in mCRC patients receiving chemotherapy, in comparison to Carcinoembryonic antigen (CEA) and Carbohydrate antigen 19-9 (CA19-9), to be used as an additional tool to computed tomography (CT). Methods: CEA, CA19-9, cfDNA concentration and cfDNA integrity index (ALU 247/115) measurements were conducted on 86 subjects divided into 43 healthy volunteers and 43 mCRC patients, before starting chemotherapy and then after 6-12 weeks of therapy initiation (3-4 cycles FOLFOX) at first response assessment. Plasma cfDNA integrity index was calculated as the ratio of long to short DNA fragments (ALU 247/115) amplified and detected by real-time PCR. Serum CEA and CA19-9 were measured by chemiluminescent immunometric assay. Results: Baseline cfDNA integrity index was statistically significantly different between responders and non-responders (p=0.03). It was found that at cut off 0.608, sensitivity was 73.7%, specificity was 66.7% and diagnostic accuracy=69.77%. Markers with statistical significant difference between responders and non-responders after chemotherapy were CEA % change (p=0.035), CA19-9 (p=0.024), cfDNA integrity index (p=0.035) and cfDNA integrity index % change (p<0.001). Among these markers, cfDNA integrity index % change had the best sensitivity (84.2%), specificity (95.2%) and diagnostic accuracy (90.7%) at cut off -17.827%. Conclusion: Baseline cfDNA integrity index can be used as a potential marker to predict response to chemotherapy. cfDNA integrity index (ALU 247/115) % change rather than its absolute value is superior to CEA, CA19-9, cfDNA concentration and their % changes in early assessment of response to chemotherapy.

Keywords: cfDNA- mCRC- cfDNA Integrity index

Introduction

Colorectal cancer (CRC) ranks as the third most common cancer globally and second in terms of mortality (Bray et al., 2018; Ferlay et al., 2018). In Egypt, CRC has been detected in 13% of patients undergoing colonoscopy, and the incidence is increasing annually with more than one-third of the cases are individuals aged 40 years and younger often diagnosed at an advanced stage (Gado et al., 2014; Brand Bateman et al., 2020).

Since 2001, the gold standard in evaluating response to chemotherapy is Response Evaluation Criteria In Solid Tumors (RECIST 1.1) which use serial CT imaging to assess changes in tumors size beside CEA and CA19-9 only if they were initially high (Somayajulu and Lieb, 2005; Rastogi et al., 2019). However, size measurement by CT focuses on just one area of the tumor, doesn’t reflect entire tumor burden and is only applied on patients with measurable lesions ≥20 mm using conventional CT or ≥10 mm by spiral CT (Berger et al., 2017; Garlan et al., 2017). It is also more accurate in late than in early response evaluation (Chao and Gibbs, 2009; Lucidarme et al., 2019).

Both Serum CEA and CA19-9 are insufficiently sensitive to be used alone for response evaluation, with their sensitivities are 68% and 81% respectively (Nicholson et al., 2014; Haque, 2019). It is therefore essential to find a new evaluation tool that can augment CT, CEA and CA19-9 for earlier assessment of response, since non-responders could be switched early to an alternative chemotherapy regimen (Okamura et al., 2017; Khakoo et al., 2018 and Lucidarme et al., 2019).

Plasma of healthy and diseased individuals have small amounts of non-cell-bound cfDNA (Jung et al., 2010). It is normally released from naturally apoptotic cells as uniformly truncated small fragments 185-200 base pair (bp) long (Umetani et al., 2006). However, tumor necrosis generates fragments >200bp (Dobrzycka et al., 2010;
Materials and Methods

The present study was conducted on 86 subjects divided into two groups

Group I included 43 mCRC patients recruited from Kasr-Alainy Hospital, Clinical Oncology and Nuclear Medicine Department during the period from February 2019 to December 2019, diagnosed by histopathological examination of tumor biopsy taken during colonoscopy/sigmoidoscopy or from the metastatic site.

Group II: included 43 healthy volunteers without significant clinical findings as controls. They were selected from volunteer blood donors at Kasr-Alainy blood bank center.

Inclusion criteria

1. Candidates for neoadjuvant chemotherapy (12 cycles FOLFOX).
2. Negative CRP to rule out any infections or inflammatory conditions.
3. Adults > 18 years old from both sexes.

Exclusion criteria

1. Patients recently received chemotherapy or radiotherapy or immunomodulatory agents.
2. Patients diagnosed with other cancers.
3. Patients having non-neoplastic conditions that may cause high cfDNA integrity index such as; sepsis, inflammations, infarctions or pregnancy.

Patients were subjected to CT assessments by RECIST criteria v1.1 at baseline (before starting chemotherapy) and after finishing the whole chemotherapy course (6-12 cycles FOLFOX taken over 12-24 weeks) to assess patient’s response to chemotherapy. Accordingly, patients were classified as having complete response (CR), partial response (PR), stable disease (SD), or progressive disease (PD). Patients with CR and PR were defined as responders, whereas those with SD and PD were defined as non-responders. Laboratory investigations (CEA and CA19-9, cfDNA extraction and determination of cfDNA integrity index) were performed before starting chemotherapy and then after 6-12 weeks of therapy initiation (3-4 cycles FOLFOX) at first response assessment.

For the controls, full history taking, clinical examination and the same laboratory investigations were performed.

This study protocol was approved by the ethical committee, Faculty of Medicine, Cairo University (Approval number: I-211018). Informed consents were taken from all participants in this study.

All laboratory work-up was performed at Clinical and Chemical Pathology Department, Routine Chemistry Unit and Molecular Biology Research and Diagnostics Unit (MRDU), Faculty of Medicine, Cairo University.

Laboratory investigations

I. Tumor markers measurement (CEA and CA19-9) using solid-phase, two-site sequential chemiluminescent immunometric assay performed on Architect i1000 SR autoanalyzer (Abbott diagnostics).

II. Molecular Studies

1) Genomic cfDNA extraction from the plasma: done using QIAamp DNA Mini Kit (QIAGEN, Germany) (Catalog number: 51304) (El-Gayar et al., 2016).
2) Measurement of cfDNA quantity and quality using Quawell Micro-volume UV-Vis Nanodrop spectrophotometer (Quawell Technology, USA): According to (Gallagher, 2001), Pure DNA used in this study had an A260 / A280 ratio of 1.7 - 1.9.
3) PCR amplification of plasma cfDNA, detection of concentration and Integrity Index:

   Cell free DNA amplification and detection were done using Applied Biosystems Step One Real-Time PCR System (Thermo Fisher Scientific Baltics UAB, V. A.). Thermo Scientific Maxima SYBR Green qPCR Master Mix (Thermo Fisher Scientific Baltics UAB, V. A.) (catalog number: K0251) was used in the assay.

   Quantitative PCR for ALU247bp and ALU 115bp was done using a standard curve created by performing qPCR on serially diluted genomic DNA obtained from Promega (Promega corporation, USA). (catalog number: G3041).

   Concentrations of the ALU fragments were measured by comparing the Threshold Cycle (CT) of the unknown sample against the standard curve with known copy numbers (Figure 1 and 2). cfDNA integrity index was

Concentrations of the ALU fragments were measured by comparing the Threshold Cycle (CT) of the unknown sample against the standard curve with known copy numbers (Figure 1 and 2). cfDNA integrity index was...
Circulating Cell Free DNA Integrity Index as a Biomarker calculated using Epi info calculator; with 0.05 alpha error and power of the study 0.80. Convenient sampling from all patients who came to Clinical Oncology department, Kasr Al-Ainy hospital during the study period with the inclusion and exclusion criteria were assigned into the study

**Statistical methods**

All statistical calculations were done using computer program IBM SPSS (Statistical Package for the Social Science; IBM Corp, Armonk, NY, USA) release 22 for Microsoft Windows.

**Results**

Forty-three mCRC patients, were 26 females and 17 males with a mean age 50.8 ± 9.8 years. The control group included 43 healthy individuals with matched age and sex. According to CT results after finishing chemotherapy using RECIST criteria v1.1 (Rastogi et al., 2019), patients with either complete response (CR) or partial response (PR) were classified as responders (44.2%), whereas patients with either stationary disease (SD) or progressive disease (PD) were defined as non-responders (55.8%).

**Comparison of studied markers in patient group before chemotherapy and control group**

The median concentration of CEA, CA19-9, baseline cfDNA and cfDNA Integrity Index were significantly higher in mCRC group than in control group (p=0.003), (p=0.019), (p=0.001) and (p=0.001) respectively (Table 1).

![Figure 1. Standard Curve for qPCR ALU 115 Primer Set (Quantity ng/ul vs Ct), Showing Slope: -3.304, Y-intercept: 12.809](image)

![Table 1. Comparison of Studied Markers in Patient Group before Chemotherapy and Control Group](image)

| Markers                      | Cases before chemotherapy (n=43) | Control group (n=43) | p-value |
|------------------------------|---------------------------------|---------------------|--------|
| CEA ng/ml                    | 19.5 (1-1500)                   | 1.9 (0.5-4.8)       | 0.003* |
| CA19-9 U/ml                  | 39.1 (1-3099)                   | 10.2 (0.6-30)       | 0.019* |
| cfDNA concentration (ALU 115) ng/ml | 502.767 (40.7-5287.1) | 82.374 (9.3-804.5) | 0.001* |
| cfDNA Integrity Index (ALU 247/115) | 0.636 (0.1-1)            | 0.178 (0-1)         | <0.001* |

*P<0.05 is considered significant
Comparison of the Studied Tumor Markers Before and After Chemotherapy in Responders and Non-Responders

The median CEA % change was -13.439% in responders and 336.5 % in non-responders. This difference was statistically significant (p=0.035). The median CA19-9 concentration after chemotherapy was statistically significantly lower in responders than in non-responders (p=0.024) (Table 2 and Figure 5).

The median baseline cfDNA Integrity Index (ALU 247/115) was statistically significantly higher in responders than in non-responders (p=0.030). The median cfDNA Integrity Index after chemotherapy was lower in responders than in non-responders with statistically significant difference (p=0.035). The median cfDNA Integrity Index % change was (-35.952 %) in responders and (18.996 %) in non-responders. This difference was statistically significant (p<0.001) (Table 2 and Figure 5).

ROC curve analysis for baseline cfDNA integrity index for predicting response to chemotherapy

The only marker that showed statistical significant difference between responders and non-responders before starting chemotherapy was baseline cfDNA Integrity Index (Table 2), so ROC curve analysis was used to detect its sensitivity and specificity in predicting response before chemotherapy (Figure 6). It showed that at cut off 0.608 (above which the patient is most likely to respond and below which the patient is most likely to non-respond).

Table 2. Comparison of the Studied Tumor Markers Before and After Chemotherapy in Responders and Non-Responders

| Markers                                | Responders (n=19) | Non-Responders (n=24) | p-value |
|----------------------------------------|-------------------|-----------------------|---------|
| CEA before (ng/ml)                     | 25.3 (1.2-1500)   | 16.35 (1-1500)        | 0.353   |
| CEA after (ng/ml)                      | 21.9 (1.1-1308)   | 171.5 (1-3-1500)      | 0.074   |
| CEA % change                           | -13.439 (-94 to1300) | 336.4 (-87.8 to14795.7) | 0.035*  |
| CA19-9 before (U/ml)                   | 64.8 (1-1835)     | 38.4 (1.2-3099)       | 0.893   |
| CA19-9 after (U/ml)                    | 53.5 (1.5-1058)   | 132.26 (1.6-1879.6)   | 0.024*  |
| CA19-9 %change                         | -26.5 (-91.9 to1697.2) | 170.807 (-82 to 4304.8) | 0.07   |
| Baseline cfDNA concentration (ALU 115) (ng/ml) | 393.692 (110.6-934.8) | 649.439 (40.7-5287.1) | 0.123   |
| cfDNA concentration (ALU 115) after (ng/ml) | 225.186 (40.8-1499.7) | 335.048 (19.9-9148)   | 0.379   |
| cfDNA concentration (ALU 115) % change | -41.35 (-89.4 to 240.3) | -35.113 (-96.8 to 298.5) | 0.366   |
| Baseline cfDNA Integrity Index (ALU 247/115) | 0.777 (0.2-1)     | 0.436 (0.1-1)         | 0.030*  |
| cfDNA Integrity Index (ALU 247/115) after | 0.477 (0.1-1)     | 0.607 (0.3-1)         | 0.035*  |
| cfDNA Integrity Index (ALU 247/115) % change | -35.952 (-80.9 to 12) | 18.996 (-19 to 352)   | <0.001* |

*Positive response if greater than or equal to

Table 3. Sensitivity and Specificity of Baseline cfDNA Integrity Index in Predicting Response to Chemotherapy in mCRC

| Area under the curve (AUC) | p value | Cut off | Sensitivity | Specificity | Positive predictive value (PPV) | Negative predictive value (NPV) | Diagnostic accuracy |
|----------------------------|---------|---------|-------------|-------------|---------------------------------|---------------------------------|--------------------|
| 0.695                      | 0.03    | 0.608*  | 73.70%      | 66.70%      | 63.64%                          | 76.19%                          | 69.77%             |

*Positive response if greater than or equal to
### Table 4. Comparison between Markers that were Significant between Responders and Non-Responders in Early Response Assessment

| Column1 | CEA % change | CA19-9 after chemotherapy | Integrity Index after chemotherapy | Integrity index % change |
|---------|--------------|---------------------------|-----------------------------------|--------------------------|
| AUC     | 0.689        | 0.702                     | 0.689                             | 0.952                    |
| P value | 0.035        | 0.024                     | 0.035                             | <0.001                   |
| Cut off*| 95.40%       | 89.4 U/ml                 | 0.533                             | -17.83%                  |
| Sensitivity |          |                           | 63.20%                             | 84.20%                   |
| Specificity |           |                           | 62.50%                             | 95.20%                   |
| PPV     | 65.22%       | 59.09%                    | 57.14%                             | 94.12%                   |
| NPV     | 80%          | 71.43%                    | 68.18%                             | 88.46%                   |
| Diagnostic accuracy | |                           | 62.79%                             | 90.70%                   |

*Positive response if less than or equal to

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![Amplification Plot](image1)

**Figure 3.** Amplification Plot of qPCR Result Obtained with Both Primer Sets for One Sample (ALU115: green line, ALU 247: purple line)

![Melt Curve ALU primer 115 and 247](image2)

**Figure 4.** Melting Curve Analysis Using Step One Real Time PCR (Blue curve shows the melting curve obtained with ALU 115 primer and purple curve shows the melting curve obtained with ALU 247 primer)
to not respond), sensitivity was 73.7%, specificity was 66.7%, PPV=63.64%, NPV=76.19% and diagnostic accuracy=69.77% (Table 3).

ROC curve analysis for markers that showed statistically significant difference between responders and non-responders in early response assessment

Markers that showed statistically significant differences between responders and non-responders after chemotherapy were CEA % change, CA19-9, cfDNA integrity index (ALU 247/115) and cfDNA integrity index (ALU 247/115) %change. Comparing the performance of the new biomarker under study, cfDNA integrity index (ALU 247/115), and the originally used markers CEA and CA19-9 in early assessment of response to chemotherapy on week 6-12 (after 3-4 cycles of FOLFOX) (Figure 7), cfDNA integrity index % change (rather than its absolute concentration) had the best sensitivity (84.2%), specificity (95.2%) and overall diagnostic accuracy (90.7%) at cut off -17.827% (i.e. ≥17.827% decrease in cfDNA Integrity index to consider the patient a responder) as shown in Table 4.

Discussion

In the current study, median baseline cfDNA concentration (ALU 115) was significantly higher in...
mCRC than in healthy controls (p=0.001). Close results were also reported by Umetan et al., (2006), Mead et al., (2011), Zaher et al., (2012), Hao et al., (2014) and Bhangu et al., (2017), Sinha et al., (2019).

Concerning its usefulness in predicting response to chemotherapy, median baseline cfDNA concentration (ALU 115) was lower in responders than in non-responders but with no statistically significant difference (p=0.123). Similar results were reported by Agostini et al., (2011) who studied 67 rectal cancer patients receiving chemoradiotherapy, found out that median baseline cfDNA concentration (ALU 115) was lower in responders compared to non-responders but with no statistically significant difference (p=0.928), therefore, baseline cfDNA concentration (ALU 115) can’t be used in response prediction.

Regarding median cfDNA concentration (ALU 115) after chemotherapy, there was no statistically significant difference between responders and non-responders (p=0.379). One explanation is stated by Jahr et al., (2001) who found out that chemotherapy can induce apoptosis of both normal and malignant cells, thus aberrantly amplifying cfDNA levels in the circulation within 48 hours, therefore, total cfDNA concentration (ALU 115) cannot be used as a marker to assess tumor response. However, in an analysis of 42 patients with advanced Non-small cell lung carcinoma (NSCLC), Kumar et al., (2010) reported that cfDNA concentration after 3 chemotherapy cycles was significantly higher in PD group than in PR (p<0.001) and SD (p = 0.001) groups.

In the current study, median cfDNA concentration (ALU 115) % change showed no statistically significant difference between responders and non-responders (p=0.366). To our knowledge there are no researches previously done for the same parameter in mCRC, however a similar result was found by Li et al., (2016) where cfDNA concentration % change did not differ significantly among NSCLC patients with different radiological response (p=0.10).

Concerning cfDNA integrity index (ALU247/115), its median level in our study was significantly higher in mCRC group when compared to control group (p<0.001). These results were in agreement with Leszinski et al., (2014) and Hao et al., (2014) who stated that cfDNA integrity was significantly higher in CRC patients compared to healthy controls (p=0.005) and (p=0.0001) respectively.

In the current study, median baseline cfDNA integrity index (ALU 247/115) was significantly higher in responders than in non-responders (p=0.303). This comes in concordance with Cheng et al., (2018) who found out that metastatic breast cancer patients who had higher baseline cfDNA integrity index showed longer Progression-free survival and overall survival than patients who had lower baseline cfDNA integrity index, (p=0.04). On the contrary, Agostini et al., (2011) found out that median baseline cfDNA integrity index in rectal cancer was lower in responsive disease compared to nonresponsive disease, however, the difference was not statistically significant. This may be due to the difference in ethnic populations.

In our study, median cfDNA Integrity Index after chemotherapy was significantly lower in responders than in non-responders (p=0.035). Similarly, Agostini et al., (2011) found out that median cfDNA integrity index after chemotherapy in 67 rectal cancer patients was statistically significantly lower in responders compared to non-responders (p=0.0009).

While assessing the utility of cfDNA Integrity Index (ALU 247/115) in early evaluation of response to chemotherapy, AUC was 0.689. and at cut off 0.553, sensitivity was 63.2%, specificity 62.5%, PPV=57.14%, NPV=68.18% and diagnostic accuracy=62.79%. Similarly, Agostini et al., (2011) found AUC=0.76, p<0.05; with a cutoff 0.44, sensitivity and specificity were 83 and 60%,
To our knowledge there is no study done regarding the prognostic significance of cfDNA integrity index % change in mCRC. However, in the present study, there was statistically significant difference between responders and non-responders (p=0.001). A ROC curve showed AUC= 0.952, and at cut-off -17.827% (i.e. at least 17.827% decrease in cfDNA Integrity index to consider the patient a responder) sensitivity was 84.2%, specificity was 95.8%, PPV=94.12%, NPV=88.46% and diagnostic accuracy=90.7%.

Concerning its usefulness in assessing tumor response to chemotherapy, our study showed no statistically significant difference in median CEA concentration before or after chemotherapy between responders and non-responders unlike CEA % change (p=0.035) with AUC=0.689, and at cut-off ≥95%, sensitivity was 78.95%, specificity 66.67% and diagnostic accuracy 72.09%. Similarly, Jia et al., (2019) stated that CEA increased in non-responders by 35% (specificity 83.33%; sensitivity 75.41%). A similar study by Kim et al., (2013), defined >20% decrease as the CEA % change cut off value with a sensitivity, specificity and diagnostic accuracy, 50.0%, 76.5% and 68.8% respectively. Also, Wang et al., (2001) stated that >50% decrease in CEA is needed for predicting positive response with sensitivity and specificity, 72% and 86% respectively. These cutoffs discrepancies could be attributed to extreme high values of CEA found in some patients leading to un-even distribution of data.

Concerning its usefulness in predicting response to chemotherapy, our study showed no statistically significant difference in CA19-9 median concentration before chemotherapy between responders and non-responders (p=0.893). To our knowledge there is no study done in this point for mCRC, but Hess et al., (2008) found out that the median overall survival for advanced pancreatic patients with higher baseline CA19-9 concentration was significantly shorter (p<0.0001), therefore he concluded that it can be an independent marker for response prediction.

In this study, CA19-9 median concentration after chemotherapy showed statistically significant difference between responders and non-responders (p=0.024) with AUC=0.702 and at cut off 89.4 U/ml, sensitivity 68.4%, specificity 62.5% and diagnostic accuracy 65.12%. To our knowledge there is no study done in this point for mCRC, however a study by Yang et al., (2013) on advanced pancreatic cancer, stated that CA19-9 is an independent prognostic factor for overall survival in which patients with a post chemotherapy CA19-9 >85.5 U/mL had significantly worse overall survival (p=0.02), and won’t benefit therapy intensification and to be considered for alternative management.

Regarding its dynamic changes before and after chemotherapy, median CA19-9 % change showed no statistically significant difference between responders and non-responders (p=0.07). Similarly, Hess et al., (2008) found out that patients who had higher % decrease in CA19-9 was not associated with a longer overall survival compared to patients who did not (p=0.74), however in a study by van Veldhuisen et al., (2018) on advanced pancreatic cancer, there was statistically significant difference in median decrease in CA19-9 between responders and non-responders (p=0.02), and ≥30% decrease in CA19-9 was defined as a cut-off with 90% sensitivity and 20% specificity. Also, Yang et al., (2013) defined 90% as a cut off for CA19-9 % change in which patients with a decrease of >90% from their baseline had a significantly improved median survival (p=0.01).

In conclusion, baseline cfDNA integrity index can be used as a potential marker to predict response to chemotherapy with sensitivity 73.7%, specificity 66.7% and overall accuracy 69.77%. cfDNA integrity index (ALU 247/115) %change is superior to cfDNA concentration (ALU 115) and the traditionally used tumor markers CEA, CA19-9 in early assessment of response to chemotherapy with sensitivity 84.2%, specificity 95.2% and overall diagnostic accuracy 90.7% at cut-off -17.827%.

Author Contribution Statement

NSE did the practical work, collected and analyzed the patients data and was a major contributor in writing the manuscript. DMA helped in the practical work. ESA provided the patients data. LAM has put the study design, validated and interpreted the patients results. AAA revised the data set. All authors read and approved the final manuscript.

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Study Approval

The study were approved by the ethical committee of the Kasr Al-Ainy School of Medicine, Cairo University as a part of NSE MD thesis.

Ethical approval

All procedures performed in the study were approved by the ethical committee of the Kasr Al-Ainy School of Medicine, Cairo University (Approval number: I-211018), with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Informed consents were taken from all participants in this study after full explanation of the study protocol.

Availability of data

The data sets used and analyzed during the current study are available from the corresponding author on reasonable request. The study was not registered in any
registering dataset.

Conflict of interest
None declared.

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