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Circulating Tumor DNA: A Biomarker for Adjuvant Treatment Response in Stage III Colon Cancer

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ABSTRACT

In this prospective work, our purpose was to evaluate the impact of circulating tumor DNA (ctDNA) analyses before, during and after adjuvant chemotherapy in completely resected stage III colon cancer patients, particularly in terms of disease-free survival (DFS). Additionally, we aimed to estimate the efficacy of adjuvant chemotherapy in decreasing ctDNA levels in the plasma. From January 2023 to December 2023, 77 patients with stage III colon cancer underwent complete surgical resection, were included in this study. These patients had undergone upfront resection of their primary tumors. All received adjuvant chemotherapy (CAPEOX or FOLFOX regimen) for six-month period. The median followup was 21 months from date of primary surgery. After six months of treatment, ctDNA levels were significantly associated with two-year DFS. Patients with low ctDNA levels exhibited better DFS compared to those with moderate ctDNA levels, who in turn had better DFS than patients with high-level ctDNA. Findings confirm the benefit of adjuvant chemotherapy in eradicating residual tumor cells responsible for tumor recurrence or metastasis.

Keywords: *Stage III colon cancer; ctDNA; ALU-247; Adjuvant chemotherapy response*

1. INTRODUCTION

According to GLOBOCAN 2022, colorectal cancer (CRC) ranked as the third most common malignancy (after lung and breast cancers), which stands for 9.6% of all cancers in both genders, and it was the second leading cause of cancer-related mortality (after bronchogenic cancer) worldwide, standing for 9.3% [1].

For resectable non-metastatic colon cancer, including stage III tumors, hemicolectomy with resection of regional lymph nodes is the preferred procedure [2]. The efficacy of adjuvant FOLFOX (5-FU/LV and oxaliplatin) in completely resected stage III colon cancer was compared to 5-FU/LV in the MOSAIC trial and results have been reported with median follow-up 114 months. Results demonstrated statistically significantly increased 5-year DFS and 10-year overall survival (OS) favoring FOLFOX arm [3]. CAPEOX (Capecitabine plus oxaliplatin) was also assessed in the adjuvant setting for stage III colon cancer in the NO16968 trial. Results of this trial showed an improved 3-year DFS, and 7-year OS compared to bolus 5-FU/L [4]. Currently, there is no accurate biomarker that can assess the patient's response to adjuvant chemotherapy, so treatment failure can not be identified till diagnosed clinical recurrence. So, the capability to figure out which patients would develop recurrence after the end of adjuvant chemotherapy would permit physicians to give them added therapy or put them under close monitoring [5].

Cell-free DNA (cfDNA) is the degraded fragments of DNA which float in the peripheral circulation. The DNA fragments which are degraded from the somatic cells, can be present in the peripheral blood and other body fluids. These fragments can have short or long chain according to the cause of cell damage. Short-chain DNA fragments of 185 - 200 base pairs (bp), are derived mainly from apoptosis and are present in the absence of any diseases. However, long-chain fragments of more than 200 bp, may be found in the circulation of patients with malignant tumors. These long chain fragments are arising from the necrosis of tumor cells underwent ischemia and surrounding tissues that are harmed by the effect of the tumor progression [6]. Plasma ctDNA is a term referred to the DNA fragments derived from malignant cells. It stands for a fraction of plasma

cfDNA, which also includes circulatory DNA that are degenerated mainly from the death of hematopoietic cells [7]. The

Arthrobacter luteus (ALU) repeat family constitutes most of plasma DNA of the normal persons. The ALU-115 primer expands short DNA fragments that are trimmed by cell apoptosis, while the ALU-247 primer expands longer DNA fragments that are trimmed by tissue necrosis. This approach specifically identifies tumor-derived DNA, particularly longer DNA fragments, which are commonly observed in malignancies [8].

ctDNA has become a potential non-invasive biomarker for cancer detection. Several studies have revealed that detection of postoperative ctDNA, is related to an elevated recurrence risk. Detection of ctDNA can be considered molecular evidence of residual disease, and the ctDNA level can be interpreted as a representative of tumor burden. ctDNA concentration analysis has an advantage of being able to be assessed serially, which enables assessment of molecular recurrence and tumor burden changes in continuous manner, to reflect the treatment response [9]. The GALAXY arm of the CIRCULATE-Japan study has analyzed ctDNA level, pre- and post-surgery in resectable stage II, III and IV CRC. After median follow-up 16.74 months, patients with positive ctDNA 4 weeks post surgery had an elevated recurrence risk (HR, 10.0) [10]. The α-CORRECT study concluded that ctDNA status determined by their patient-specific, tumor-informed ctDNA assay was a strong prognostic elemen for risk of recurrence in stage III CRC patients, at all timepoints. The prognostic relation between ctDNA status and tumor recurrence was greater than other clinical factors, including CEA, which is the current standard of care [11].

2. AIM OF THE STUDY

Evaluation of the frequency of postoperative low, moderate, and high ctDNA levels in stage III colon cancer patients and its effect on DFS, as well as analyzing the effect of adjuvant chemotherapy for stage III colon cancer, on reducing ctDNA levels, at two time points; post 3 and 6 months of adjuvant therapy.

3. PATIENTS AND METHODS

Patients enrolled in this study had been matched with the following criteria: (1) valid informed consent form, (2) ≥ 18 years old, (3) Eastern Cooperative Oncology Group (ECOG) Scale ≤ 2, (4) underwent complete surgical resection of the colonic tumor, (6) Histologically confirmed diagnosis with adenocarcinoma of the colon, (6) Stage III tumor, defined by AJCC cancer staging, 8th edition.

Study Design

Pathology was reviewed with reference pathologist to confirm diagnosis with stage III colonic adenocarcinoma. Radiological studies were performed using magnetic resonance imaging (MRI) or computed topography (CT) scan of the abdomen and pelvis with contrast, and CT chest, to detect any residual disease or distant metastasis, to all patients prior to start of adjuvant therapy.

Assessment before starting treatment included full medical history and complete physical examination. Further investigations conducted within 2 days before every chemotherapy cycle included vital signs, ECOG performance status, complete blood count (CBC) and liver and kidney function tests.

ctDNA was done postoperatively (2-4 weeks after surgery before starting adjuvant chemotherapy), post 3-month treatment with adjuvant chemotherapy and after completing adjuvant chemotherapy (post 6-month treatment). Patients were classified according to the ctDNA level into three groups: low-level (<500 ng/ml), moderate-level (500-1000 ng/ml) or high-level (>1000 ng/ml).

Patients underwent adjuvant chemotherapy with either the CAPEOX or FOLFOX regimen for a duration of six months. The adjuvant chemotherapy regimens employed in our study were as follows: modified FOLFOX-6, which consisted of oxaliplatin 85 mg/m2 and folinic acid 200 mg/m2 administered intravenously on day 1 and 15, followed by a 5-FU 400 mg/m2 intravenous bolus and subsequently a 5-FU 2,400 mg/m2 intravenous infusion over 48 hours, administered every four weeks. CAPEOX, on the other hand, consisted of oxaliplatin 130 mg/m2 administered intravenously on day 1, followed by oral capecitabine 1000 mg/ m2 administered every 12 hours from day 1 to day 14, and repeated every three weeks.

Levels of ctDNA for all patients were measured according to mean and median parameters to estimate the percentage of ctDNA clearance after treatment with adjuvant chemotherapy. Clearance of ctDNA is defined as the change of ctDNA level after treatment (=$Post\ therapy\ ctDNA - Baseline\ ctDNA$). Percentage of ctDNA clearance is defined as ctDNA clearance level in proportion to the baseline ctDNA level (=[$ctDNA\ clearance / Baseline\ ctDNA$]%).

Follow-up of the patients conducted post adjuvant chemotherapy and every three months thereafter, using laboratory and radiological evaluation to detect local disease recurrence or distant metastasis (disease related). Laboratory evaluation included tumor makers (CEA and CA19-9). Radiological evaluation included CT scan of the chest, abdomen, and pelvis.

Molecular Studies

Peripheral venous blood samples (5-10 ml) from every patient were obtained in K2 EDTA 10 ml tubes and were processed within 2 hours at molecular pathology unit at NCI Cairo. Plasma was isolated from blood samples by double centrifugation.

First tube was centrifuged by 2000 rpm for 10 minutes. Supernate was aspirated and transferred to a new centrifuge tube. Plasma was centrifuged again by 13,000 rpm for 10 minutes, to remove any cells or organelles. Supernate was aspirated and transferred to a cryotube and stored at -80 °C.

DNA was produced from 200 µL of plasma utilizing QIAamp® blood DNA Mini extraction kit (Qiagen, Hilden, Germany) as reported by the reference manual of the manufacturer. DNA was eluted in 50 µL elution buffer and concentration was determined by Nanodrop spectrophotometer. Extracted DNA was stored at -80 °C. PerfectStart SYBR Green Kits (ready-to-use kit) was used including ROX as reference dye. Primers' sequences for ALU-247; F: 5′-GTGGCTCACGCCTGTAATC-3′, R: 5′-CAGGCTGGAGTGCAGTGG-3′. For each sample, a master mix was prepared consisting of SYBR Green, Rox dye, primers and ctDNA template. Components and volumes are shown in table (1).

Table (1): Master mix components and volumes

Component	Volume
SYBR green master mix	10 µL
ALU-247 forward primers	0.4 µmol/L
ALU-247 reverse primers	0.4 µmol/L
Rox dye	0.1 µL
DNA template	4 µL
Ultra pure water	5.1 µL

ctDNA was amplified and quantified using the Applied Biosystems 7500 Fast real-time PCR system. The procedure began with a heat activation step for DNA polymerase at 94 °C for 30 seconds. This was followed by 45 amplification cycles, each consisting of denaturation at 94 °C for 5 seconds, annealing at 60 °C for 15 seconds, and extension at 72 °C for 10 seconds. To verify the specificity of the PCR products, a melting curve analysis was performed after amplification.

To generate standard ALU-247 curve, 7 serial dilutions (from 10000 ng/mL to 0.001 ng/mL) of human genomic DNA control PCR-ready concentration (Promega) were amplified by qPCR. They were used as reference to quantify ALU-247 concentration in each sample, relative to the standard curve. For each sample, cycle threshold (CT) value of ctDNA was recorded. The CT value was plotted in the standard curve and concentration of ctDNA was calculated using the standard curve equation.

Ethical Statement

Ethical approval for this prospective study was aquired from Institutional Review Board (IRB) of National Cancer Institute (NCI), Cairo University, on 13 November 2022. IRB Approval No. 2210-305-042. All participants signed informed written consent prior to enrollment in the study.

4. STATISTICAL METHODS

Analysis of this data was done by utilizing SPSS win (statistical package of social science) version 28. Numerical data was presented as appropriate, including means, medians, standard deviations, and ranges. Categorical data was presented in the form of percentages and frequencies. A comparative analysis of two numerical datasets was conducted employing either the student's t-test or the Mann-Whitney U test, dependent on the appropriateness of each test for the data characteristics. A paired "t" test was employed to differentiate two related groups of normally distributed variables, while a Wilcoxon test was utilized to compare non-normally distributed numeric variables. A repeated measures ANOVA was employed to compare multiple related groups of normally distributed numerical data. A comparison of multiple related groups of non-normally distributed numerical variables was performed using the Friedman test. A comparative analysis of categorical data was conducted utilising either the Chi-Square or Fisher's exact test, dependent on the specific nature of the data.

Kaplan-meire method was utilized to estimate the DFS. The follow-up duration was computed from the date of primary surgical intervention to the date of metastasis or relapse. Statistically significant differences in survival curves were measured using the log-rank test. Cox regression analysis was conduted to evaluate independent prognostic variables which affect the survival time. All tests were managed with the two-tailed approach, and a probability (P) value less than 0.05 was deemed statistically significant.

5. RESULTS AND DISCUSSION

ctDNA Groups

The distribution of patients across low-, moderate-, and high-level ctDNA groups shifted dynamically in response to adjuvant

chemotherapy. Prior to chemotherapy, low-level group constituted a smaller subset of the study population. It expanded significantly following 3 months of therapy. It grew further to encompass a substantial majority of patients by the completion of treatment. In contrast, moderate-level group showed a transient increase in representation at the 3-month interim assessment. But subsequently declined to a minimal proportion post-treatment. High-level group initially comprised the largest fraction of the cohort. It experienced a steady reduction in prevalence over the course of chemotherapy. Figure (1) clarify these changes.

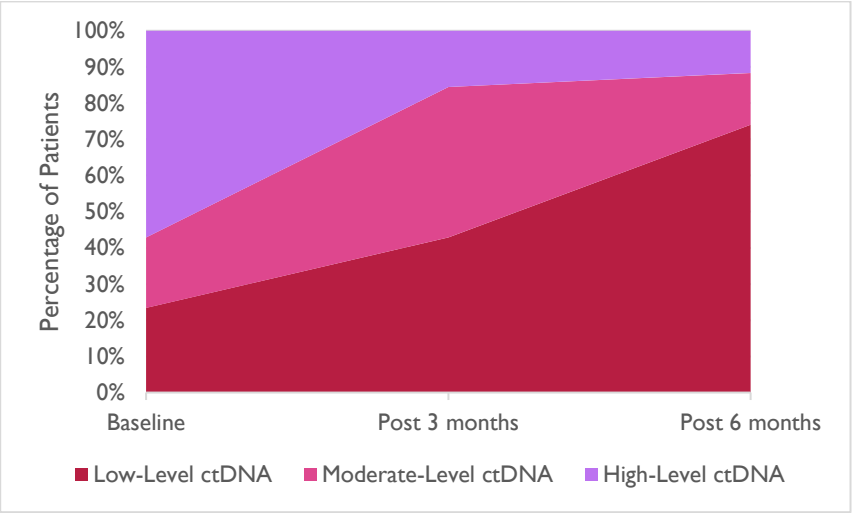

Fig. 1: ctDNA groups over the treatment period

ctDNA Clearance

The high mean and median ctDNA values, before starting adjuvant chemotherapy suggest that a significant proportion of patients may have higher risk of recurrence. After adjuvant treatment for 3 months, the decreased mean and median values suggests that adjuvant chemotherapy is effective in reducing ctDNA levels, which is a positive indicator of treatment response and potentially lower risk of recurrence. The further decrease in mean and median ctDNA values after treatment for 6 months indicates that the adjuvant chemotherapy regimen is effective in reducing ctDNA levels over time. Comparison between treatment for 3 versus 6 months regarding ctDNA clearance, revealed that the added benefit for the second 3 months of treatment was equal to the first one in terms of median values (ctDNA clearance = -39%), and to less extent in terms of mean values (ctDNA clearance = -23 vs -34%, respectively). Mean and median values are illustrated in figure (2), and numerical values are detailed in table (2).

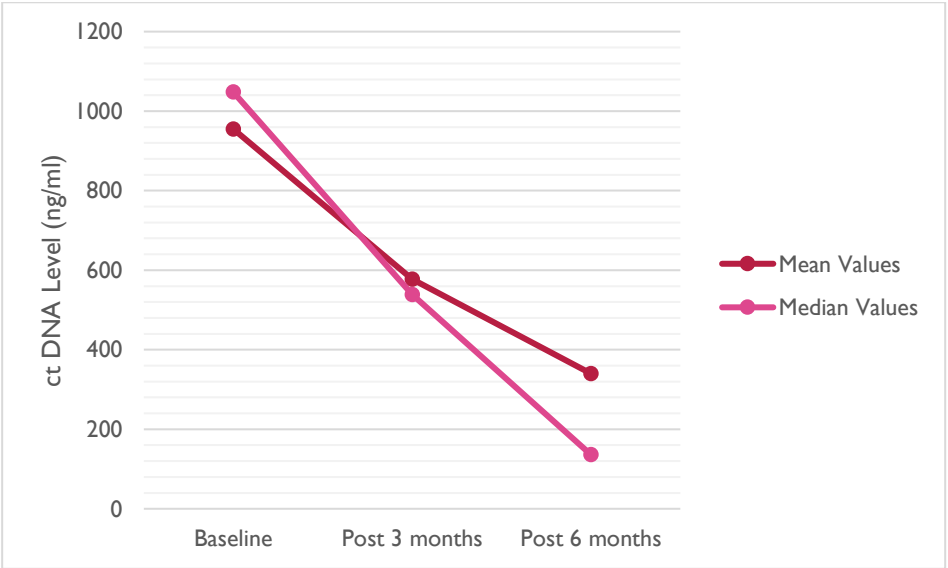

Fig. (2): ctDNA values before, during and after adjuvant chemotherapy

Table (2): Values of ctDNA at the three timepoints

		Total N = 77
ctDNA at Baseline (ng/ml)	Mean ± SD	955.32 ± 528.7
	Median (IQR)	1048 (618 – 1500)
	Range	0 – 1500
ctDNA Post 3-month treatment (ng/ml)	Mean ± SD	577.21 ± 415.12
	Median (IQR)	539 (218 – 860)
	Range	0 – 1500
ctDNA Post 6-month treatment (ng/ml)	Mean ± SD	340.34 ± 430.23
	Median (IQR)	136 (0 – 513)
	Range	0 – 1500
ctDNA clearance post 3-month treatment (%)	Mean ± SD	-34.19 ± 49.19
	Median (IQR)	-39.39 (-59.07 – -20)
	Range	-100 – 114.08
ctDNA clearance post 6-month treatment (%)	Mean ± SD	-57.04 ± 61.55
	Median (IQR)	-78.44 (-100 – -44.6)
	Range	-100 – 182

Relation between ctDNA Level and DFS

The analysis of this study demonstrated that, prior to initiating adjuvant chemotherapy, ctDNA levels were not statistically significant predictors of 2-year DFS. However, a significant association emerged after 3 months of treatment (P=0.001) and after 6 months of treatment (P<0.001). Notably, the low-ctDNA group exhibited a superior DFS rate compared to the moderate-ctDNA group, which in turn demonstrated a better DFS rate than the high-ctDNA group. Figures (3), (4) and (5) illustrate the difference in survival. These findings suggest that adjuvant chemotherapy may be effective in eliminating residual tumor cells responsible for tumor recurrence or metastasis. These data are in accordance with Malla et al., who reported that ctDNA has been shown to have several promising implementations, including minimal residual disease detection (MRD), predicting early relapse, and assessment of response to treatment [12]. Two studies by Tie et al. and Henriksen et al. reported that analysis of ctDNA level postoperatively and post adjuvant chemotherapy is a potential marker for prognosis in patients with stage III colon cancer [13, 14].

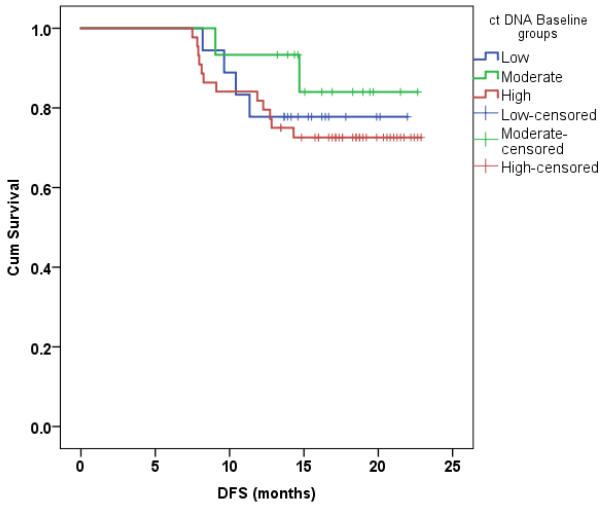

Fig. (3): Disease free survival among baseline ctDNA groups

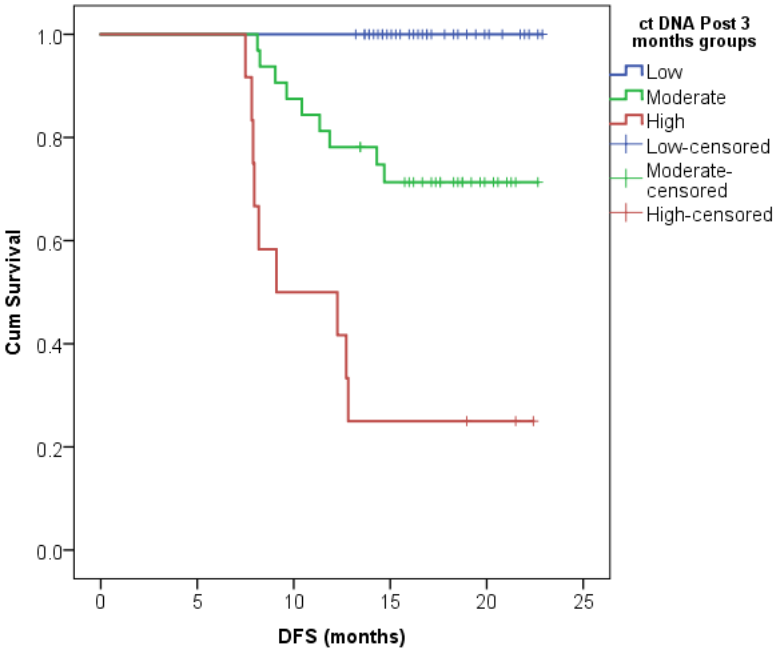

Fig. (4): Disease free survival among post 3-month ctDNA groups

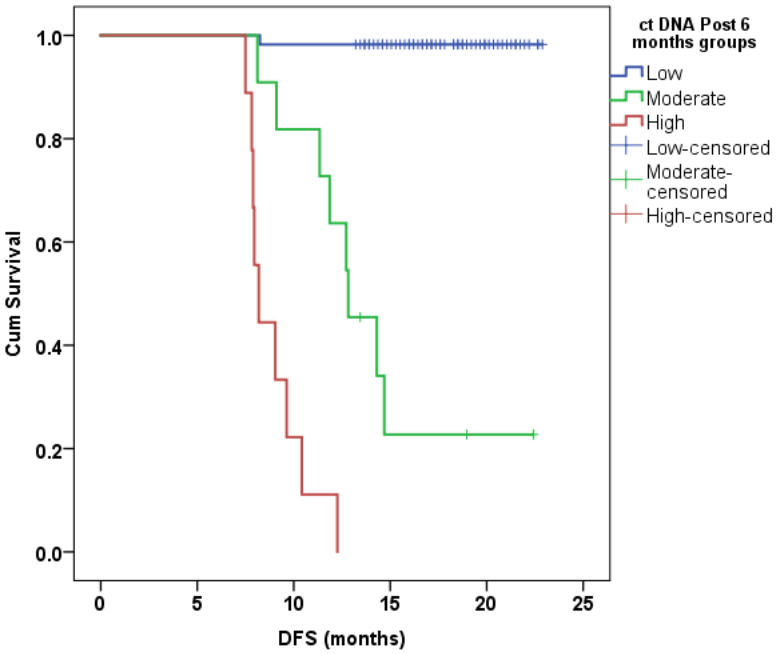

Fig. (5): Disease free survival among post 6-month ctDNA groups

6. CONCLUSION

In this prospective work, our aim was to estimate the efficacy of treatment with adjuvant chemotherapy on decreasing ctDNA level in plasma of patients with completely resected stage III colon cancer. Also, we aimed to measure the impact of ctDNA level, on DFS.

During the period between January 2023 and December 2024, 77 patients with completely resected stage III colon cancer, were presented to the medical oncology outpatient clinic at NCI Cairo and Maadi Armed Forces Medical Complex, after upfront resection of primary tumor. They received adjuvant chemotherapy for 6 months, and they were followed up thereafter. Median followup was twenty-one months (range 18-24 months) from date of surgery.

The analysis of this study revealed that after treatment for 3 and 6 months, ctDNA levels were significantly associated with 2-year DFS. After 3- and 6-month treatment with adjuvant chemotherapy, low-ctDNA patients had better DFS than moderate and high levels of ctDNA patients. Also, moderate-ctDNA patients had better DFS than high levels of ctDNA patients. This data may reflect the effect of adjuvant chemotherapy to eradicate the residual tumor cells responsible for tumor recurrence or metastasis.

7. CONFLICTS OF INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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