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Caspase-Cleaved Cytokeratin 18 as a Potential Molecular Biomarker for Monitoring Chemotherapeutic Response in Breast Cancer Patients

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Abstract

Background: Breast cancer is one of the most dreadful cancer types, with the highest mortality and morbidity in women in both low and high-income countries. Cytokeratins can be applied as noninvasive, efficient and satisfactory molecular tools to monitor and predict the response to chemotherapy for breast cancer.

Objective: This study was intended to explore the potential application of ccCK18 as a molecular biomarker for monitoring and predicting the efficacy of chemotherapy on breast cancer patients.

Methodology: A hospital-based prospective study was conducted on 40 breast cancer patients and 38 apparently healthy control individuals in Black Lion Specialized Hospital. Blood samples were obtained from study subjects and control groups before chemotherapy, at 4 and 6 hours after chemotherapy. An ELISA assay was applied to measure plasma caspase-cleaved Cytokeratin 18 (ccCK18). The association between expression of ccCK18 and the tumor metastasis and stages and grades were determined with ELISA. Different biochemical tests were also carried out to investigate the function of liver in relation to ccCK18 level with respect to cancer chemotherapy. Wilcoxon signed rank test, Spearman's rho test and paired t-test were applied as statistical tools to determine association and correlation among different the study parameters. A *p*-value of <0.05 was considered as statistically significant.

Results: The baseline levels of plasma ccCK-18 were significantly higher in patients with breast cancer than those in the control group (% CI= 95%, p<0.05). The level of ccCK-18 was also significantly increased at 6 hours after chemotherapy ($p<0.05$). Patients with pT3 tumor size displayed the highest median level compared to other tumor sizes. The ccCK-18 level was observed to be higher among patients with distant metastasis than in non-metastatic patients. Lactate dehydrogenase (LDH) levels were also elevated at 6 hours, following chemotherapy. Plasma liver function tests (ALT, AST, ALP and total bilirubin) were normal before and after chemotherapy, indicating that there was no major liver damage following chemotherapy.

Conclusion: ccCK-18 level in blood could be used as a molecular biomarker for monitoring the disease and predicting the response of patients to breast cancer chemotherapy, particularly in low settings; however, further studies with other protocols are warranted to tailor chemotherapy treatment in a better way.

Keywords: Breast cancer; Caspase cleaved cytokeratin-18; Biomarker; Chemotherapy

Abbreviations: 5-FU: 5-Fluorouracil; AC: Adriamycin and cyclophosphamide; AKT: *Ataxia-Telangiectasia*; AMP: Amino Methyl Propanol; ccCK-18: Caspase-cleaved Cytokeratin18; CK: Cytokeratin; DNA: Deoxyribonucleic Acid; ELISA: Enzyme Linked Immunosorbent Assay; FAC: 5-Fluorouracil, Adriamycin and Cyclophosphamide; IF: Intermediate Filament; IQR: Interquartile Range; LDH: Lactate Dehydrogenase; NAD: Nicotinamide Adenine Dinucleotide; SCLC: Small Cell Lung Cancer; BLSH: Black Lion Specialized Hospital; TMB: Tetra-MethylBenzidine; TNM: Tumor Node Metastasis

Introduction

Breast cancer is a leading cause of morbidity and mortality for women and affects more than a million patients annually [1,2]. Chemotherapy, mastectomy and radiotherapy are some of the common treatment modalities available for breast cancer. After applying one of these therapeutic modalities, monitoring the progress of the disease and effectiveness of the treatment with noninvasive strategies is crucial. This can be facilitated by analyzing the expression pattern of different molecular biomarkers in the biological samples of the patient. For instance, one of the mechanisms how chemotherapy kills cancer cells is by inducing apoptosis (programmed death of cells). The induction of apoptosis in tumor cells by the given chemotherapy can be monitored and quantified by the trends of expression of cytokeratins in a given patient sample [3-5].

Cytokeratins (CKs) are one of the key molecular biomarkers that are applied to follow up how the patient is responding to a given treatment. They are part of the cytoskeleton and the largest family of proteins comprising of more than 20 intermediate filaments that are expressed in cells of epithelial and endothelial origin [6,7]. CKs are composed of complexes of one type I and one type II CK proteins that become organized into larger filamentous polymeric structures. The common example of the heteropolymer cytokeratin complex is the combination

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of CKs 8 and 18. Cytokeratin 8, CK18 and CK19 are expressed by most types of carcinomas, including the breast [4,8], prostate [9], ovary [10], lung [11] and colon cancer [12].

Under normal physiological conditions cytokeratins are complexed in intermediate filament of epithelial cells and remain insoluble. Cancer cells in the stage of proliferation contain a substantial pool of soluble CKs (CK8, 18 and 19), which can increase in response to stress. The levels of circulating CKs are significantly increased in patients with epithelial cell-derived malignancies [13,14]. Most type I CKs display caspase cleavage sites allowing for the detection of apoptotic cell death through assessment of neo-epitope in the process of their specific degradation. Release of CK18 into the soluble pool occurs through remodeling of the intermediate filaments in the case of necrosis opposite to apoptosis, in which intermediate filament proteins (including CK18) are targeted by activated caspases 3, 7 and 9 to facilitate the formation of apoptotic bodies [9]. When apoptosis is induced, CK 18 is cleaved from aspartate amino acids localized at position 238 and 396. The caspase-cleaved cytokeratin 18 can be detected by simple enzyme linked immunoprecipitation assay (ELISA) method with the aid of a monoclonal antibody M30. This newly formed neo-epitope of CK18 can be considered as a selective molecular biomarker of apoptosis that is induced by the given chemotherapy, indicating that it is useful for the estimation of the efficacy of the given therapy [13,14]. Therefore, the current study was intended to investigate the potential role of caspase-cleaved cytokeratin 18 as a molecular biomarker for monitoring chemotherapy response of breast cancer patients in Ethiopian female patients.

Materials and Methods

Study design, sample collection sites and study population

A prospective comparative study was conducted on female breast cancer patients at Black Lion Specialized Hospital (BLSH). This study was conducted on 40 previously untreated, histopathologically confirmed breast cancer patients and 38 apparently healthy control individuals. Age greater than 18 years and ability of patients to comply with the study procedure were also considered as an inclusion criterion. Patients with liver disorder, renal dysfunction and sepsis were excluded from the study. In addition, pregnant and lactating women, and patients attending systemic anticancer therapy or radiotherapy were also excluded. Samples were collected under aseptic condition and standard operating procedure (SOP).

Sample collection and plasma sample preparation: Blood samples of study participants were collected prior to chemotherapy (baseline, at 0 hour), at 4 and 6 hours after the treatment. The same samples from the control group were collected once during the study period. The patients were treated based on standard protocol: six cycles of 5-fluorouracil, adriamycin and cyclophosphamide (FAC) or four cycles of adriamycin and cyclophosphamide (AC) regimens. All drugs were administered on day 1 and repeated at every 21 days. A 5mL of blood sample was drawn from the antecubital vein of the study subjects and collected into EDTA tubes (Improvacuter, Guangzhou, china). Samples were inverted gently several times and kept for no longer than 30 min until centrifugation at 1000g for 10 min. The upper plasma layer was transferred to eppendorf tube (Eppendorf™, Fisher Scientific, USA) and frozen upright at -20°C before transferring to -80°C for long-term storage.

Clinico-pathological records of the breast cancer patients: Clinic-pathological characteristics of the study subjects was obtained from the medical recorders of the patients at oncology department, Black Lion Specialized Hospital. The records of pathological staging, grading, metastasis, and lymph node involvement of the study subjects was obtained to explore its impact on the expression pattern of ccCK in breast cancer patients before and after chemotherapy. Thus, the level of ccCK in these patients was correlated with these pathological characteristics. *p*-values less than 0.05 is considered as statistically significant.

Quantification of caspase-cleaved cytokeratin 18 with ELISA assay: The M30 Apoptosense ELISA is a solid-phase sandwich enzyme immunoassay that is a powerful tool for detecting and quantifying a specific protein. It is a one-step *in vitro* immunoassay for the quantitative determination of the apoptosis-associated caspase-cleaved cytokeratin 18 (ccCK18) in serum and plasma. This technique was used to quantify M30-antigen (ccCK-18) by using M30-Apoptosene ELISA kit that was obtained from PEVIVA-VLVbio (Stockholm, Sweden). The measurement was done according to the instructions of the manufacturer (PEVIVA-VLVbio, Stockholm, Sweden). Briefly, the ELISA machine was calibrated to adequate temperature and appropriate wavelength prior to the actual measurement. A standard curve was prepared before the measurement of test samples. Blood samples were collected in EDTA tubes to obtain serum from 40 female breast cancer patients and 38 control groups. After coagulating for 15 min at room temperature, serum was obtained by centrifugation at 1000g for 10 min. The upper plasma layer was transferred to eppendorf tube (Eppendorf ™, Fisher Scientific, USA) and stored at -20°C till needed for analysis shortly. Twenty-five microliters of standards, controls (positive and negative) and test samples were added to a 96-well microtiter plates that were coated with an M30 antibody. While shaking at 600 RPM, the microtiter well was allowed to be incubated for 4 hours. Then, the microtiter plate was washed five times with an automatic ELISA plate washer (BioTek, ELx50 Washer, USA) using a specific washing buffer. Next, 200 µL of tetra-amino benzidine was added to microtiter well and incubated in a dark environment for 20 minutes. Finally, 50 µL of stopping solution (1M sulphuric acid) was added to the micro titer plate and allowed to stay for 5 minutes before reading with ELISA reader. Then, the absorbance was measured within 30 minutes with an ELISA microplate reader (Thermo lab Systems, Multiscan, UK) at 450 nm and the ccCK-18 levels were estimated. The concentration of the ccCK-18 was expressed as unit per liter (U/L). All measurements were performed by senior laboratory technicians blinded to all clinical data of the study subjects. Assays were performed at the Central Laboratory of the Black Lion Specialized Hospital (Addis Ababa University, Ethiopia). Each ELISA was repeated at least three times and standards, samples, blanks and controls were analyzed in triplicates. p-values less than 0.05 is considered as statistically significant.

Evaluation of liver function with biochemical tests: This study was also intended to assess and compare different liver function tests (ALT, AST, ALP, and bilirubin) before and after chemotherapy to rule out liver damage as a cause of plasma cytokeratin 18 elevation. To this end, alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase and lactate dehydrogenase were analyzed by fully automated clinical chemistry analyzer (Mindray clinical chemistry analyzer, China). All reagents used for these tests were bought from BioSystems (Barcelona, Spain). Total bilirubin was also analyzed by cobas integra automated clinical chemistry analyzer (Cobas integra 400 clinical chemistry analyzer, Germany) and the reagents were obtained from Sigma Aldrich. All of the tests were done by experienced senior laboratory technicians blinded to all clinical data of the study at the Central Laboratory of the BLSH (Addis Ababa University, Ethiopia). The tests were done under standard operating procedures (SOP) and

according to the instruction of manufacturers. Each test was repeated at least three times and in triplicates. p-values less than 0.05 is considered as statistically significant.

Statistical analysis

Statistical analysis was performed using Statistical Package for the Social Sciences (SPSS) *Version 21.0* (SPSS Inc., Chicago, IL, USA). Graphpad Prism *version 6* (Graphpad Software Inc., California, US) was used to draw graphs. Wilcoxon Signed Rank Test and paired *t-test* was used to determine mean and interquartile range differences of ccCK18, liver function markers and LDH values between samples of the breast cancer patients before and after chemotherapy, and the patients and the corresponding normal subjects. *p*-values less than 0.05 were considered as statistically significant.

Results

Socio-demographic characteristics of the study population

All of the study subjects were female breast cancer patients. The age range of study population and control groups was 48 (minimum 20 and maximum 68) and 36 (22 to 58), respectively. The mean age of breast cancer patients and healthy (non-breast cancer) participants was 40.8 ± 9.5 and 36.6 ± 7.5 years, respectively. Among 40 breast cancer patients, 37 (92.5%) were married, 2 (5.0%) single and 1 (2.5%) was divorced. Eighteen (45.0%) of the study participants had 1-3 children, 11 (27.5%) had 4-6 children, 7 (17.5%) had no child and 4 (10.0%) had more than 7 children. The general socio-demographic characteristics of the study participants were outlined in Table 1.

Clinico-pathological characteristics of study participants

From 40 breast cancer patients, 5 (12.5%) had a pT1 (tumor size ≤ 2 cm diameter), 15 (37.5%) had pT2 (tumor size 2 to 5 cm diameter), 10 (25.0%) had pT3 (tumor size > 5 cm diameter) and 8 (20.0%) had pT4 (tumor of any size but with extension to chest wall or skin). A quarter of study subjects were presented with pN2, 8 (20.0%) were presented with pNx, 10 (25.0%) were presented with pN1, 2 (5.0%) were presented with pN0 and 8 (20.0%) were presented with pN3. Sixteen (40.0%) had no distant metastasis while 14 (35.0%) of the study subjects had no distant metastasis assessed during pathological diagnosis. Patients who had distant metastasis were only 8 (20.0%). Invasive ductal carcinoma was the most common of all others in our study subjects (75%), followed by invasive lobular carcinoma (20%) (Table 2).

Control groups	
Age (mean ± SD) (years)	36.6 ± 7.5
Sex	All Women
Breast cancer patients	
Age (mean ± SD) (years)	40.8 ± 9.5
Sex	All Women
Marital status, *N (%)*	
Single	2 (5.0%)
Married	37 (92.5%)
Divorced	1 (2.5%)
Number of children, N (%)	
0	18 (45.0%)
1-3	11 (27.5%)
4-6	7 (17.5%)
≥7	4 (10.0 %)
Note: Number of Control groups, N=38 and Number of study subjects, N=40.	

Table 1: Socio-demographic characteristics of breast cancer patients in Ethiopia.

Degree of differentiation, N (%)	
Poorly differentiated	11 (27.5 %)
Moderately differentiated	16 (40.0 %)
Well differentiated	12 (30.0 %)
Unspecified	1 (2.5 %)
Histological types of the tumor, N (%)	
Invasive ductal carcinoma	30 (75.0 %)
Invasive lobular carcinoma	8 (20.0 %)
Unspecified	2 (5.0 %)
TNM Classification of the tumor, N (%)	
Tumor Size	
pT1	5 (12.5 %)
pT2	15 (37.5 %)
pT3	10 (25.0 %)
pT4	8 (20.0 %)
Unspecified	2 (5.0 %)
Lymph Node Status	
pNx	8 (20.0 %)
pN0	2 (5.0 %)
pN1	10 (25.0 %)
pN2	10 (25.0 %)
pN3	8 (20.0 %)
Unspecified	2 (5.0 %)
Distant Metastasis	
Mx	14 (35.0 %)
cM0(i+)	16 (40 %)
M1	8 (20.0 %)
Unspecified	2 (5.0 %)
Note: Number of patients analyzed for each parameter, N = 40.	

Table 2: Clinico-pathological characteristics of breast cancer patients in Ethiopia.

ccCK-18 (U/L)	Control	Predose (0 hour)	4 hours post chemotherapy	6 hours post chemotherapy
Median	187.3	238.7	241.5	268.4
Minimum-Maximum	101.4-423.5	213.2-1003.2	210.0-1057.1	216.0-1108.7
25th to 75th Percentile	140.0-220.9	228.7-303.0	230.61-321.4	245.0-340.1
p-Value		$<0.05^{\alpha}$	$>0.05^{\beta}$	$<0.05^{\gamma}$
Note: α = *p*-value calculated against control group, β = *p*-value calculated against predose, γ = *p*-value calculated against predose				

Table 3: Pre and post chemotherapy plasma caspase-cleaved cytokeratin 18 level in breast cancer patients and control groups.

Caspase-cleaved cytokeratin 18 after chemotherapy

The ccCK18 level was recorded in all study subjects at different time interval. Accordingly, the median and interquartile range (IQR) at baseline (0 hour) was 238.7 and 228.7-303.0 U/L, respectively. The median and IQR ccCK18 level after 4 hours chemotherapy were 241.5 and 230.6-321.4 U/L, respectively. The median and IQR ccCK18 level after 6 hours chemotherapy were 268.4 and 245.0-340.1 U/L, respectively. Spearman's rho test showed that the values of ccCK18 were independent of age (p = 0.160, p = 0.064 and p = 0.191, for baseline, 4 and 6 hours post-chemotherapy, respectively). The median and IQR reference values for ccCK18 level in control groups were 187.3 and 140.0-220.9 U/L, respectively. The Wilcoxon Signed Rank Test indicated that the expression of ccCK18 levels increased 6 hours after chemotherapy ($p < 0.05$, CI = 95.0%). In addition, ccCK-18 level at 4 hours following chemotherapy was still higher than the baseline level; but it was not statistically significant ($p > 0.05$). Moreover, the median level of ccCK18 was significantly lower in apparently healthy control than in breast cancer patients prior to chemotherapy (187.3

U/L vs 238.7 U/L, Wilcoxon Signed Rank Test, % CI = 95%, $p < 0.05$) (Table 3).

Caspase-cleaved cytokeratin 18 levels and distant metastasis and tumor size

Plasma caspase-cleaved cytokeratin 18 levels of breast cancer patients with different tumor size, and distance metastasis has been assessed and compared to each other. The median and IQR level of baseline ccCK18 was higher in patients with metastatic breast cancer than in patients with non-metastatic breast cancer. It was also found that patients with metastatic breast cancer express higher ccCK18 level than patients with metastatic status could not be assessed (Mx). In addition, there was statistically significant correlation between ccCK-18 and distant metastasis (Spearman's rho test correlation coefficient = 0.435, $p = 0.032$).

The expression level of ccCK18 was determined for breast cancer patients at different stages of the disease. Comparison of ccCK18 level in breast cancer patients' sample at pre-chemotherapy and post-chemotherapy indicated significant variation of expression. The median and IQR level of ccCK18 4 hours after chemotherapy was higher among patients with metastasized breast cancer (M1) than in patients with no metastasis, and in patients whose metastasis status could not be assessed (Mx). The median and IQR level of ccCK-18 six hours after chemotherapy was found to be higher among patients with distant metastasis than in patients with no metastasis and patients with non-assessable metastatic status (Figures 1A-1C).

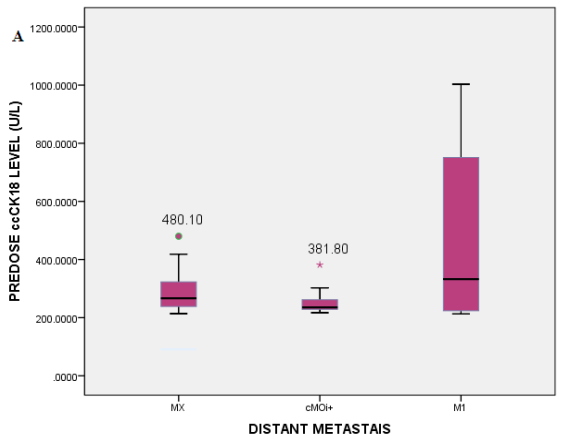

Figure 1A: Baseline plasma ccCK-18 level in breast cancer patients with different metastatic status.

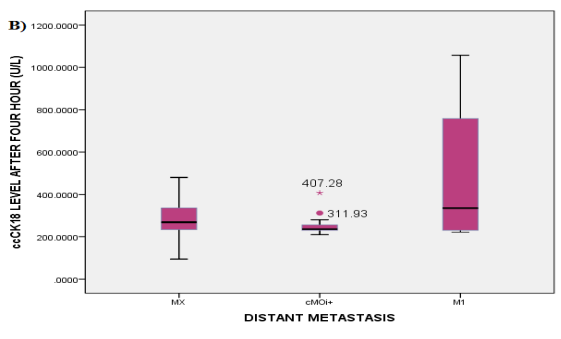

Figure 1B: Plasma ccCK-18 level in breast cancer patients with different metastatic status 4 hours after chemotherapy.

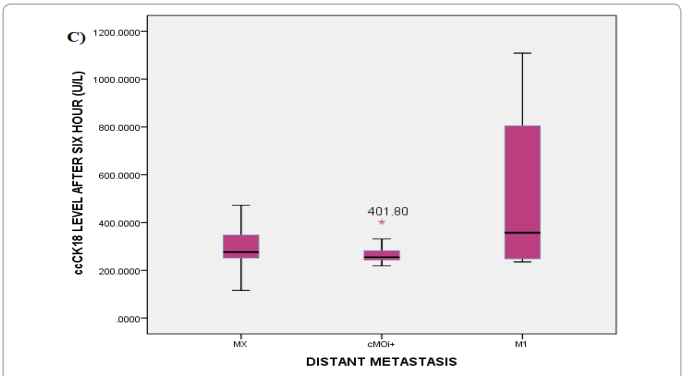

Figure 1C: Plasma ccCK-18 level in breast cancer patients with different metastatic status 6 hours after chemotherapy.

Comparison of the levels of ccCK-18 with different clinico-pathological risk factors revealed higher median difference with the type of tumor size. It was found that patients with pT3 tumor size seem to have the highest plasma ccCK-18 levels. The median and IQR level of ccCK18 was higher in patients with pT3 tumor size than in patients with pT1, pT2 and pT4 tumor size. Patients with pT1 tumor size have lowest median level of ccCK18 level followed by pT4 tumor size. Furthermore, Spearman's rho test showed that the baseline ccCK-18 was correlated with the tumor size (Correlation coefficient = 0.326, $p = 0.04$) (Table 4).

Caspase-cleaved cytokeratin 18 level and plasma markers of liver inflammation

This study was to investigate whether serum ccCK-18 level is associated with plasma markers of liver injury. None of the liver function indictor proteins expression [aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP) and total bilirubin] was linked with plasma ccCK 18 levels. Correlation analysis between plasma LDH and ccCK-18 level was assessed but there was no significant association between the two parameters found (Table 5) summarizes all correlation analysis of plasma ccCK-18 with liver function markers as well as plasma tumor marker-LDH. The study carried out on liver function test with biomarker proteins indicated that chemotherapy had no impact on the liver toxicity. There were also no patients with elevated plasma concentrations of these liver function markers after 4 or 6 hour post-chemotherapy. There was no significant change in LDH level 4 hours post-chemotherapy; however, a significant increase in plasma LDH level was observed 6 hours post-chemotherapy (Table 5).

The effect of different drug regimen on the level of plasma ccCK-18 was assessed. There was no significant difference in plasma ccCK-18 between breast cancer patients who had taken 5-flourouracil, adriamycin and cyclophosphamide and adriamycin and cyclophosphamide regimen (median ccCK-18 = 265.1 U/L) and AC regimen (median ccCK-18 = 237.4 U/L) at 4 hours post chemotherapy ($p = 0.481$, Spearman's rho test). Similarly, there was no significant difference in plasma levels of ccCK-18 among breast cancer patient who had received 5-flourouracil, adriamycin and cyclophosphamide regimen (median ccCK-18 = 269.2 U/L) and adriamycin and cyclophosphamide regimen (median ccCK-18 = 255.5 U/L) at 6 hours post chemotherapy (p = 0.401, Spearman's rho test) on their first cycle.

Discussion

Apoptosis is one of the mechanisms in which chemotherapeutic

	Caspase-cleaved Cytokeratin 18 Level (U/L) Median (25^{th}-75^{th} percentile)		
Tumor Size	**Predose (0 Hour)**	**4 hours post chemotherapy**	**6 hours post chemotherapy**
pT1	222.8 (152.0-245.2)	224.1 (158.1-244.0)	248.3 (175.4-261.2)
pT2	251.8 (231.3-276.2)	250.1 (232.6-278.1)	262.9 (242.6-331.5)
pT3	364.8 (232.4-480.1)	371.4 (230.5-480.1)	410.3 (255.3-472.3)
pT4	230.7 (221.4-301.2)	239.1 (233.0-320.7)	259.4 (245.9-315.6)

Table 4: Tumor size and caspase-cleaved cytokeratin-18 level in breast cancer patients before and after chemotherapy.

Variables	Pre-dose (0 Hour)		4 hours post chemotherapy		6 hours post chemotherapy	
	Correlation Co-efficient	*p*-value*	Correlation Co-efficient	*p*-value*	Correlation Co-efficient	*p*-value*
AST	0.069	0.67	0.087	0.594	-0.01	0.952
ALT	-0.014	0.934	-0.015	0.926	-0.121	0.457
ALP	0.018	0.915	-0.010	0.945	-0.067	0.681
T. Bilirubin	0.069	0.673	0.109	0.504	0.110	0.499
LDH	0.016	0.922	0.041	0.799	-0.095	0.558

Table 5: Relationships of caspase cleaved cytokeratin-18 level with other biochemical parameters.

Variables	Control (Mean ± SD)	Predose (Mean ± SD)	4 Hour (Mean ± SD)	*p*-Value*	6 Hour (Mean ± SD)	*p*-Value*
AST (U/L)	19.4± 8.8	19.3± 9.7	18.8 ± 10.6	0.743	19.8 ± 11.2	0.465
ALT (U/L)	14.7 ± 6.4	14.1± 8.6	14.8 ± 7.9	0.830	16.1± 7.8	0.435
ALP (U/L)	181.1± 36.1	182.8 ± 37.3	181.6± 36.9	0.828	183.1± 39.2	0.446
T.Bilirubin (mg/dL)	0.494 ± 0.2	0.527 ± 0.3	0.524 ± 0.3	0.928	0.526 ± 0.8	0.976
LDH (U/L)	284.0 ± 111.1	352.5 ± 157.0	381.9± 240.1	0.250	550.2± 488.0	0.037
Note: AST, Aspartate Aminotransferase; ALT, Alanine Aminotransferase; ALP, Alkaline Phosphatase; T. Bilirubin, Total Bilirubin. *Comparison of AST, ALT, ALP, total bilirubin and LDH levels before chemotherapy and those 4 hours and 6 hours after chemotherapy, Paired t test, where $p<0.05$ is statistically significant.						

Table 6: Pre and post chemotherapy liver function tests and LDH level in breast cancer patients and normal controls.

agents kill tumor cells. It is a form of regulated cell death that is characterized by specific structural changes in cells and mediated by proteases of the caspase family [15]. Caspase enzyme activity itself or the presence of specific cell degradation products facilitated by caspases can be applied for the detection of apoptosis in tumor cells. One of the crucial molecules released from cells during chemotherapy-induced caspase-facilitated apoptosis is M30-antigen or ccCK-18. This protein is released into the blood stream of the patient and applied as a predictive molecular marker in monitoring cancer chemotherapy [16]. This study was intended to explore the potential of ccCK18 as a novel molecular biomarker for monitoring chemotherapeutic response in Ethiopian breast cancer patients. Here, we demonstrated that plasma ccCK-18 level was elevated in the serum of breast cancer patients prior to chemotherapy compared to control groups. Recent research report indicated that tumorigenesis can induce apoptosis and high frequency of apoptosis drives cancer metastasis. This could be due to the nutritional, immunological and metabolic pressure in the tumor microenvironment [17]. Our finding mirrors several other research results in different types of cancers. Elevated level of ccCK-18 and total cytokeratin 18 (tCK18) was reported in colorectal cancer patients [8,12,18]. Other studies also revealed higher expression of ccCK-18 and tCK18 level in testicular cancer [19]. and pancreatic cancer [20,21]. Furthermore, it was shown that this protein was also highly expressed in small cell lung cancer [11], breast cancer [4,8]. and advanced gastric carcinoma [8,22,23]. More interestingly, a study done among colorectal cancer patients revealed that ccCK-18 levels were higher in tumor-draining venous samples compared with peripheral sampling [18]. Taken together, the finding in this study supports the hypothesis that the apoptotic index of tumor tissues is much higher than that of normal tissues. Therefore, it is important to determine the baseline apoptotic value before considering the intermediate and the end products of chemotherapy-induced apoptosis as a predictive molecular biomarker.

The current study demonstrates that there was time-dependent rising expression of chemotherapy-induced ccCK-18 level in breast cancer patients. In similar fashion, preclinical study done by Cummings and his colleagues, on vascular disrupting agents to treat cancers, showed a time and dose-dependent increase of ccCK-18 at 6 hours post chemotherapy. There was not observed significantly increased expression of ccC18 in both our study and others at 4 hours post-chemotherapy [24]. Here, we clearly showed the variation in expression pattern of ccCK18 as a function of time after chemotherapy. Taken together, ccCK18 is a promising predictive molecular biomarker in breast cancer patients taking chemotherapy. Moreover, this study also supports a rational hypothesis for evaluating serial blood ccCK18 secretion, using a recently developed assay as a promising non-invasive molecular biomarker in operable breast cancer where patients routinely receive anthracycline-containing regimen for 3-6 months depending on type of drug combination [25].

It is highly possible that the stage or the total size of the tumor mass seems to affect the ccCK-18 levels in plasma. In the present study, we investigated the plasma level of ccCK18 in pre-and post-chemotherapy breast cancer patients in relation to the tumor size. Our finding indicated that the median plasma levels of ccCK18 before and after chemotherapy differed with tumor size. Patients with bigger tumor size, particularly, the pT3 showed a higher median level of ccCK-18 at both before and after chemotherapy (Table 4). Unlike pT3, the breast cancer patients with pT4 tumor size were found to have a lowest level of ccCK-18 than pT2. The higher expression of ccCK18 level in pT3 breast cancer patients before chemotherapy is attributed to the harsh tumor microenvironment [26], whereas the elevated level of the molecule after chemotherapy is linked with chemotherapy-induced apoptosis. The lower ccCK-18 level in pT4 tumor sizes as compared to pT2 and pT3 tumors before chemotherapy may be due to the fact that pT4 tumors correspond to tumors of any size that are characterized by extension to the chest wall or skin therefore there could be lesser physiological

pressure in this particular tumor microenvironment. In contrast, the pT1, pT2 and pT3 tumor sizes have limited space and metabolic support in the tumor microenvironment which increases the apoptotic pressure. Therefore, ccCK18 levels in pT4 tumors cannot be compared directly with pT1, pT2 and pT3 tumors before chemotherapy.

Altogether, there must be a close link between apoptosis and both malignancy itself and tumor size and extent of metastasis.

Furthermore, investigation demonstrated that the expression of ccCK-18 level was dependent on distant metastasis. Here, we show the median and IQR levels of baseline plasma ccCK-18 was higher in the metastatic than the non-metastatic groups. This implies that the aggressiveness (metastatic ability) of tumor mass may have an impact on the plasma level of ccCK-18. It can be explained by the fact that most of tumor cells at metastatic stage are found in the apoptotic pool than that of proliferative pool because of the limiting factors in the tumor stroma. Similarly, Olofsson and his colleagues showed that breast cancer patients with metastasis have higher median ccCK-18 level than no metastasis. Many other studies also revealed a significant difference in the mean level of ccCK-18 between metastatic breast cancer patients and locally advanced ones (4,5). Studies on gastrointestinal adenocarcinoma also revealed elevated extent of ccCK-18 at baseline in patients with metastatic disease compared with those with locally advanced disease (8). The post-chemotherapy investigation of the status of ccCK18 indicated higher expression of the molecule in the metastatic breast cancer group than non-metastatic ones. This may be a reflection of the extent of disease present and potential for access to the circulatory system, as patients with metastatic disease had higher baseline levels of ccCK-18 than those with locally advanced disease. This suggests that the baseline plasma ccCK-18 levels may give an indication of both tumor burden and also the amount of cell death that is occurring whether this is as a result of chemotherapy or as part of the ongoing disease process.

Hepatocytes also express cytokeratins and liver toxicty induced by cancer therapeutics can evoke release of additional cytokeratins. As a proof-of-principle, we analyzed the liver function surrogate markers in our study subjects at pre-and post-chemotherapy. Accordingly, there was no association observed in plasma concentrations of liver function test markers (ALT, AST, ALP and total bilirubin) and the ccCK18 after chemotherapy. The absence of a liver dysfunction and protracted elevation of serum ccCK-18 concentrations after chemotherapy was also reported by Greyskote and his colleagues (18). Altogether, it confirms that the tumor cell rather than the liver is the origin of post-chemotherapy increase in ccCK-18 levels among cancer patients who had no potentially ill conditions in their liver function at the start of chemotherapy (Table 6).

According to our finding, there was no correlation observed between plasma LDH and ccCK-18 in breast cancer patients after chemotherapy. On the other hand, the team of de Haas reported strong relationship between ccCK-18 level and LDH among testicular cancer patients receiving chemotherapy regimen consisting of bleomycin, etoposide, and cisplatin. This disparity could be due to the differences among the site of the carcinoma studied, the chemotherapy applied, the stages of the disease or the experimental protocols applied by the two studies.

Conclusion

Improving the efficacy and reducing the toxicity of cancer chemotherapy are amongst the biggest challenges facing cancer control. Therefore, noninvasive detection of ccCK-18 level in blood sample of breast cancer patients before and after chemotherapy is an invaluable molecular tool to monitor the efficacy of chemotherapy in breast cancer patients. That is, ccCK-18 level in plasma could be used as molecular biomarker and a predictive marker of response in breast cancer patients with measurable breast cancer disease, particularly in low settings where there is no state-of- the art molecular laboratories; however, further studies with other protocols are warranted to tailor chemotherapy treatment in a better way.

Competing Interests

We, the authors, declare that we have no competing interests.

Author's Contribution

Yohannes Gemechu and Daniel Siefu conceived the idea and designed the study. Wajana Lako Labisso and Daniel Seifu analysed the data. Wajana Lako Labisso, Yohannes Gemechu wrote the manuscript. Yohannes Gemechu, Daniel Seifu, and WondemagegnTigneh carried out patient recruitment plan and sample collection. Daniel Seifu, WondemagegnTigneh and Wajana Lako Labisso reviewed and revised the manuscript for important intellectual content. All authors read and approved the final manuscript for publication.

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Ethical Considerations

Ethical clearance was obtained from Addis Ababa University, College of Health Sciences, Department of Biochemistry Ethical Review Committee prior to the study. In addition, written consent was obtained from the study participants.

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