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EXPRESSION OF ALU REPEAT IN BLOOD PLASMA OF PATIENTS WITH BREAST CANCER DURING NEOADJUVANT CHEMOTHERAPY: AN EXPLORATORY STUDY

Background. Locally advanced breast cancer (LABC) rates are unusually high in developing countries. There is a need for the identification of predictive biomarkers for the selection of patients who could benefit from neoadjuvant chemotherapy (NAC). **Aim.** As the expression of ALU repeat is increased in cancer and has not been assessed in liquid biopsy of cancer patients, our goal was to assess ALU expression in the blood plasma of LABC patients during NAC. **Patients and Methods.** Plasma samples drawn at baseline and at the end of the fourth cycle of chemotherapy were used to determine the plasma levels of ALU-RNA by quantitative real-time PCR. **Results.** ALU expression from baseline to the fourth cycle of NAC increased from a median relative level of 1870 to 3370 in the whole group ($p = 0.03$). The increase in ALU-RNA levels in the course of NAC was more pronounced in premenopausal women and in patients with hormone-positive tumors. In patients with complete response to NAC, baseline ALU expression was higher than that in those with partial response. **Conclusion.** This exploratory study provides evidence that plasma ALU-RNA levels are modulated by the menopausal status and hormone receptor status of breast cancer patients and pre-therapeutic ALU-RNA levels might be useful in predicting the response to chemotherapy in a neoadjuvant setting.

Keywords: locally advanced breast cancer, neoadjuvant chemotherapy, ALU expression, menopausal status.

Breast cancer (BC) is the most common cancer in women worldwide [1] and its incidence and mortality rates tend to increase in developing countries [2]. In Turkey, BC incidence has increased almost twofold over the last 2—3 decades from 24 cases per 100,000 in 1994 to 50 cases per 100,000 in 2013 [3].

Locally advanced breast cancer (LABC) represents tumors that are inoperable or operable only by mastectomy at the initial presentation

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and includes large breast tumors (>5 cm) or tumors with extensive lymph node involvement [4]. LABC rates are unusually high in developing countries as multiple factors including a lack of awareness and the absence of national screening programs impact the diagnosis [5]. Given the advanced presentation of the disease, the prognosis of LABC is relatively poor with median survival rates ranging from 28 to 66 months reported in different studies [6, 7] and a 5-year locoregional recurrence rate of 7—9% [8, 9]. Neoadjuvant chemotherapy (NAC) is the primary option to treat the locally advanced disease for tumor down-staging and to control loco-regional recurrence rates. Improved response to NAC correlates with better survival outcomes [10].

The selection of patients who could benefit from NAC is of clinical relevance [11]. As BC is a heterogeneous disease, often histologically similar tumors respond differentially to therapy with some molecular subtypes having higher rates of the pathological complete response (pCR) to NAC while others do not have pCR. Molecular markers may be beneficial to determine avoidable treatments with associated toxicities for patients who are not responsive to NAC [12]. Recent developments in the fields of genomics and transcriptomics facilitate the identification of novel molecular biomarkers to be implemented into clinical practice [13]. Moreover, liquid biopsy-based detection and characterization of circulating tumor DNA and other cancer-associated molecular biomarkers in bodily fluids offer new opportunities in the management of cancer patients.

ALU sequences are the most abundant short interspersed repeat elements (SINEs) constituting 11% of the human genome and tending to be located in gene-rich regions [14]. In physiologic conditions, these elements are transcribed by RNA polymerase III at low levels as their transcription is restricted by tight epigenetic silencing via DNA methylation. However, under different circumstances such as cancer, ALU expression is increased [15]. As ALU expression has not been assessed in liquid biopsy in cancer patients and in those with BC, this descriptive study aimed to assess ALU expression in the blood plasma of patients with LABC during NAC. MALAT1, a non-repeat non-coding RNA, was used as a control molecule in this study design.

Patients and Metods

Forty archive plasma samples drawn from patients between May 2015 and June 2016 were included in the study. The vast majority of patients in the cohort had invasive ductal carcinoma (IDC) with locoregional lymph node involvement. Some of the patients' characteristics are shown in the Table. Peripheral blood samples were obtained at baseline and at the end of the fourth cycle of NAC, which included adriamycin and cyclophosphamide (4 cycles) and paclitaxel regimes (12 cycles). The data on the expression of hormone receptors, Her2, and Ki-67 along with other clinical characteristics were retrieved from patients' files. The pathological complete response (pCR) was defined as the complete disappearance of invasive disease in the breast and in axillary lymph nodes by radiological evaluation. The Review Board of the Oncology Institute (Turkey) approved the study.

Total RNA was extracted from plasma samples (each 200 μL) using the RNA isolation reagent (Roche, Germany) according to the instructions. The purity analysis of plasma RNA was done on a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, USA). Using the First-Strand cDNA Synthesis kit (Thermo Scientific, USA), RNAs were converted to cDNAs, which served as a template for the amplification of ALU and MALAT1 specific RNAs in plasma. The PCR measurements were performed using a Roche LC80 instrument with SYBR Green as a fluorescence molecule. The relative plasma levels of ALU-RNA and MALAT1 were determined by the

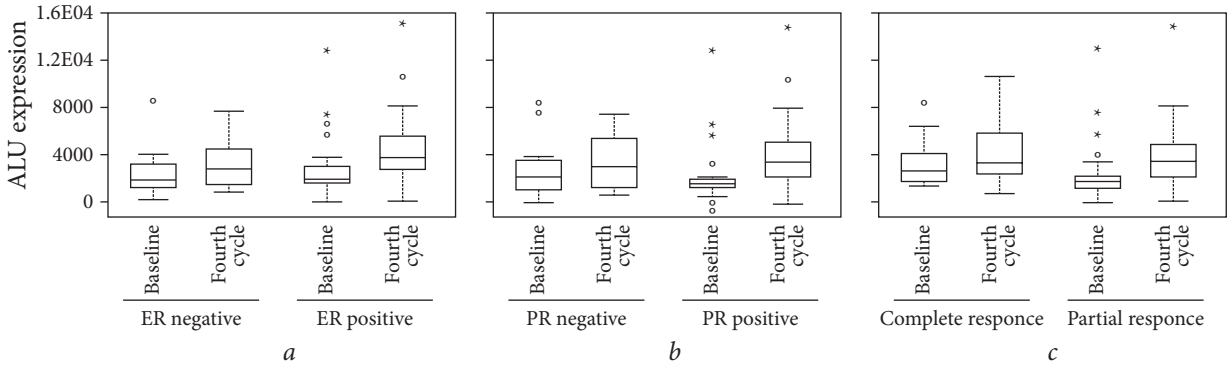

Figure. ALU-RNA levels by the menopausal and hormone receptor status and by the response to neoadjuvant chemotherapy (NAC): *a* — plasma ALU-RNA levels in the course of NAC by the estrogen receptor expression; *b* — plasma ALU-RNA levels in the course of NAC by the progesterone receptor expression; *c* — plasma ALU-RNA levels in the course of NAC in patients with complete or partial response to NAC. ER — estrogen receptor; PR — progesterone receptor

Table. **Demographic and clinicopathological characteristics of the BC patients**

Variable	n
Age	
<40 (27–40)	21
≥41 (41–68)	19
Menopausal status	
Premenopausal	26
Postmenopausal	14
T stage	
T1–T2	14
T3–T4	26
Ki-67 score	
<20	8
≥20	32
Prognostic classification	
Hormone-positive	30
Triple negative	5
Her2-positive (luminal and non-luminal)	22
Pathological response to NAC	
Complete	9
Non-complete	31

$2^{-\Delta Ct}$ formula with *GAPDH* as the internal control. The following primer pairs were used: ALU-F (5'-3') CAACATAGTGAAACCCCGTCTCT, ALU-R (5'-3') GCCTCAGCCTCCCGAGTAG; MALAT1-F (5'-3') GGATCCTAGACCAGCATGCC, MALAT1-R (5'-3') AAAGGTTACCATAAGTAAGTTCCAGAAAA; GAPDH-F (5'-3') AGCCACATCGCTCAGACAC, GAPDH-R (5'-3') GCCCAATACGACCAAATCC.

We employed the Mann — Whitney U test to assess the association of the plasma RNA levels of ALU and MALAT1 with clinicopathological variables. The Wilcoxon signed-rank test was used to test the differences between baseline and fourth cycle levels of ALU-RNA and MALAT1 in plasma. $p < 0.05$ was considered as the level of statistical significance.

Results and Discussion

As ALU expression is increased in cancer [15], we evaluated its utility as a liquid biopsy marker in patients with LABC during NAC. First, we assessed the association between plasma ALU-RNA levels and clinicopathological variables of the BC patients. Only menopausal status was found to be associated with ALU expression; the pre-therapeutic plasma ALU-RNA Table

were significantly lower in premenopausal women (median 1700) than in postmenopausal women (median 2850) ($p = 0.04$). In contrast, the pre-therapeutic MALAT1 levels were comparable in premenopausal (median 0.13) and postmenopausal (median 0.16) women ($p = 0.6$). As the exact mechanisms that led to the accumulation of ALU-RNA in cancer cells are not known, our finding suggests a suppressive role of estrogens in ALU expression. It is possible that hormones modulate the epigenetic silencing of ALU sequences, which tend to become unmethylated in cancer cells [16]. Further research is needed to explore the effect of estrogens on ALU expression.

Next, we investigated the plasma ALU expression at baseline and at the end of the fourth cycle of NAC and found that the plasma ALU-RNA levels increased from baseline (median 1870) to the end of the fourth cycle of NAC (median 3370) ($p = 0.03$) in the whole group whereas MALAT levels decreased. The subgroup analysis revealed that the increase in ALU-RNA levels from baseline to the end of the fourth cycle was mainly due to samples from the premenopausal women (from 1700 at baseline to 3741 at the end of the fourth cycle) ($p = 0.003$). In contrast, in the postmenopausal women, a small decline of ALU-RNA from 3120 to 2854 ($p = 0.7$) was evident. Similarly, the increase in median ALU-RNA levels was more evident in hormone-positive patients than in hormone-negative patients (Figure, *a* for estrogen and Figure, *b* for progesterone). On the other hand, in patients with pCR, according to radiological evaluation, baseline ALU expression was higher than that in those with partial response (2550 *vs.* 1780, Figure, *c*) suggesting that higher baseline levels of ALU-RNA might be an indicator of the response to NAC. The value of ALU expression in predicting the response to NAC should be evaluated in further studies with larger sample sizes.

In conclusion, this is the first study on liquid biopsy-based evaluation of ALU expression and provides evidence that the menopausal status of breast cancer patients and hormone receptor status of breast tumors affect the plasma levels of ALU-RNA in the course of cytotoxic therapy. As ALU-RNA accumulates in most premenopausal patients and in patients with hormone receptor-positive tumors during the chemotherapy course, we speculate that the killing of hormone receptor-bearing tumor cells by chemotherapy results in the upregulation of ALU expression in plasma.

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ЕКСПРЕСІЯ ALU-ПОВТОРІВ У ПЛАЗМІ КРОВІ ХВОРИХ НА РАК МОЛОЧНОЇ ЗАЛОЗИ В ПРОЦЕСІ НЕОАД'ЮВАНТНОЇ ХІМІОТЕРАПІЇ: ПОШУКОВЕ ДОСЛІДЖЕННЯ

У країнах, що розвиваються, рівень захворюваності на місцево-поширений рак молочної залози (МРМЗ) надзвичайно високий. Існує необхідність в ідентифікації прогностичних біомаркерів, які дозволять виокремити пацієнтів, чутливих до неоад'ювантної хіміотерапії (НАХТ). **Мета.** Дослідити рівні експресії ALU в плазмі крові пацієнтів з МРМЗ під час НАХТ. **Пацієнти та методи.** Визначення рівня ALU-РНК у плазмі крові пацієнток, відібраної перед початком лікування та в кінці четвертого циклу хіміотерапії, проводили за допомогою кількісної ПЛР у реальному часі. **Результати.** Встановлено зростання середнього відносного рівня експресії ALU від 1870 на початку лікування до 3370 після четвертого циклу НАХТ (p = 0,03). Підвищення рівня ALU-РНК у процесі НАХТ було більш вираженим у жінок з пременопаузою та пацієнток з гормон-позитивними пухлинами. Встановлено достовірно вищі рівні ALU у хворих із повною відповіддю на НАХТ порівняно з аналогічними показниками хворих із частковою відповіддю на терапію. **Висновки.** Результати проведеного дослідження свідчать про наявність асоціативних зв'язків між рівнем ALU-РНК у плазмі крові пацієнток та їхнім репродуктивним статусом, а також експресією рецепторів стероїдних гормонів у тканині новоутворень. Перспективним є використання показників експресії ALU-РНК для прогнозування відповіді на НАХТ.

Ключові слова: місцевопоширений рак молочної залози, неоад'ювантна хіміотерапія, експресія ALU, менопаузальний статус.