

Liquid biopsy utilizing miRNA in patients with advanced breast cancer treated with cyclin-dependent kinase 4/6 inhibitors

MARCIN KUBECZKO¹, PATRYCJA TUDREJ², TOMASZ TYSZKIEWICZ², ALEKSANDRA KRZYWON³,
MAŁGORZATA OCZKO-WOJCIECHOWSKA² and MICHAŁ JARZĄB¹

¹Breast Cancer Center; Departments of ²Clinical and Molecular Genetics and ³Biostatistics and Bioinformatics,
Maria Skłodowska-Curie National Research Institute of Oncology Gliwice Branch, Gliwice, Upper Silesia 44-102, Poland

Received October 17, 2023; Accepted January 26, 2024

DOI: 10.3892/ol.2024.14314

Abstract. Cyclin-dependent kinase 4/6 inhibitors (CDK4/6is) are the mainstay of treatment of hormone receptor⁺/human epidermal growth factor receptor 2⁻ patients with advanced breast cancer (ABC). Despite improvements in overall survival, most patients experience disease progression. Biomarkers derived from a liquid biopsy are appealing for their potential to detect resistance to treatment earlier than computed tomography imaging. However, clinical data concerning microRNAs (miRNAs/miRs) in the context of CDK4/6is are lacking. Thus, the present study assessed the use of miRNAs in patients with ABC treated with CDK4/6is. Patients treated for ABC with CDK4/6is between June and August 2022 were eligible. miRNA expression analyses were performed using a TaqMan[™] low-density miRNA array. A total of 80 consecutive patients with ABC treated with CDK4/6is at Maria Skłodowska-Curie National Research Institute of Oncology (Gliwice, Poland) were assessed, with 14 patients diagnosed with progressive disease at the time of sampling, 55 patients exhibited clinical benefit from CDK4/6i treatment and 11 patients were at the beginning of CDK4/6i treatment. Patients with disease progression had significantly higher levels of miR-21 (P=0.027), miR-34a (P=0.011), miR-193b (P=0.032), miR-200a (P=0.027) and miR-200b (P=0.003) compared with patients who benefitted from CDK4/6i treatment. Significantly higher levels of miR-34a expression were observed in patients with progressive disease than in patients beginning treatment (P=0.031). The present study demonstrated the potential innovative role of circulating miRNAs during CDK4/6i treatment. Plasma-based expression of miR-21, -34a, -193b, -200a and -200b effectively distinguished

patients with ABC who responded to CDK4/6i treatment from patients who were resistant. However, longitudinal studies are required to verify the predictive and prognostic potential of miRNA.

Introduction

Breast cancer is the most frequently diagnosed cancer worldwide, with >2.3 million new cases diagnosed in 2020 (1), and its management has improved over the past decade (2). Cyclin-dependent kinase 4/6 inhibitors (CDK4/6is) are the mainstay of treatment of hormone receptor (HR)⁺/human epidermal growth factor receptor 2 (HER2)⁻ patients with advanced breast cancer (ABC) in first- and second-line settings. Despite improvements in progression-free survival (PFS) and overall survival (OS), as well as quality of life, most patients develop disease progression due to drug resistance, resulting in poor prognosis. Drug resistance is a common phenomenon; thus, effective breast cancer therapy depends on appropriately monitoring the patient response to treatment (3).

In ABC trials, recurrent diagnostic imaging has been used as often as every 6-12 weeks (4). Response Evaluation Criteria in Solid Tumors (RECIST) 1.1, in which lesions are described with imaging, is widely accepted as a standardized measure of tumor response to therapy (5). Nonetheless, disease may progress with an ineffective therapy for a considerable period before further imaging is performed. Furthermore, given prolonged PFS in patients with ABC, imaging is time-consuming and toxicity of iodine-based and gadolinium contrast may be an issue (6).

Tools based on tissue biopsies are not appropriate for permanent monitoring, due to their invasive nature. Liquid biopsies overcome these obstacles as sampling is quick, minimally invasive and associated with low-risk complications. Furthermore, circulating biomarkers can be collected more often than imaging or tissue sampling, theoretically enabling more informed management. Liquid biopsy comprises circulating tumor cells and DNA and microRNA (miRNA/miR). Serum biomarkers are appealing for the potential to detect resistance to treatment and disease progression earlier than computed tomography (CT) imaging and circulating miRNAs, such as miR-96 and miR-125, reflect presence of breast tumors (7).

Correspondence to: Dr Marcin Kubeczko, Breast Cancer Center, Maria Skłodowska-Curie National Research Institute of Oncology Gliwice Branch, 15 Wybrzeże Armii Krajowej Street, Gliwice, Upper Silesia 44-102, Poland
E-mail: marcin.kubeczko@gliwice.nio.gov.pl

Key words: breast cancer, CDK4/6 inhibitor, liquid biopsy, microRNA

miRNAs are small (18-25 nucleotides) non-coding, single-stranded RNA molecules that interact with specific target messenger (m)RNAs, thus instigating their translational repression or degradation. miRNAs modulate gene expression by binding to a complementary sequence in the 3'untranslated region of the mRNA (8). miRNAs may be dysregulated in cancer due to genetic (genomic amplification, chromosomal rearrangements, deletions or mutation) and epigenetic changes (aberrant hypermethylation, global DNA hypomethylation and post-translational histone modification) (9). Genome-wide analyses have demonstrated that dysregulated expression of miRNAs contributes to the pathogenesis of almost all types of human malignant diseases, and >30% of genes are direct targets of miRNAs (10,11). A single miRNA molecule can regulate hundreds of mRNAs. The association between miRNA and cancer is not entirely understood. Multiple feedback loops, numerous targets of single miRNA and the fact that several miRNAs control the same mRNA, result in an intricate web of relationships (9).

miRNAs are key regulators of cancer-related pathways, and there is growing evidence that miRNAs serve an essential role in response to CDK4/6is (12,13). Certain miRNAs are associated with sensitivity to CDK4/6is, whereas others confer resistance to treatment with CDK4/6is. Let-7a is upregulated in luminal breast cancer (14), whereas circulating miR-17 and miR-34a levels differ between patients who are HR⁻ and HR⁺ (15). Hyperactivation of the PI3K/AKT/mTOR pathway is common in T cell acute lymphoblastic leukemia (ALL)/lymphoma. CDK6 is one of the most downregulated targets of let-7 and miR-21 in mTOR knockdown tumors and treatment with an mTOR inhibitor (rapamycin) combined with palbociclib is effective (16). Epigenetic downregulation of miR-9 induces upregulation of CDK6, while treatment of ALL cells with palbociclib decreases proliferation and increases apoptosis (17). The miR-17-92 family includes miR-17, -19a and -20a (18). The E2F1-regulated miR-17-92 cluster is highly expressed in proneural glioblastoma, which exhibits increased vulnerability to CDK4/6 inhibition, and the E2F cell cycle pathway may be a key driver. Palbociclib decreases expression of the miR-17-92 family in sensitive glioblastoma cell-like lines by suppressing E2F1 transcription factor (19). miR-29b inhibits breast cancer cell proliferation and increases sensitivity to palbociclib (13). miR-106b expression is efficiently suppressed by CDK4/6 inhibition in an E2F and retinoblastoma-dependent manner (20). Silencing of the tumor suppressor miR-124a regulates CDK6 expression and confers poor prognosis in ALL (21). Palbociclib decreases ALL cell proliferation *in vitro*, whereas overexpression of pre-miR124a in a mouse model leads to decreased tumorigenicity (21). miR-126 is involved in cell cycle regulation, particularly M phase, and improves the effects of ribociclib *in vitro* (22). In the aforementioned study, miR-326 was reported to have a non-significant anti-proliferative effect as a single agent and conferred sensitivity to ribociclib. Furthermore, miR-223 may serve as an oncosuppressor and an oncopromoter. miR-223 expression is decreased in luminal breast cancer and inversely correlated with survival of patients. Moreover, E2F1 is a suppressor of miR-223 transcription. Therefore, CDK4/6i, by inhibiting E2F1 activity, may reinstate miR-223 expression and breast cancer resistance to CDK4/6 inhibition induced

by miR-223 abrogation, both *in vitro* and *in vivo* (23,24). Additionally, serum-based miRNA signature, composed of miR451a, miR-16-5p, miR-17-3p and miR-940, effectively distinguishes patients who respond to the first-line combination of chemotherapy and trastuzumab from patients who are resistant (25).

However promising, most studies concerning miRNAs in the context of CDK4/6is are preclinical (21,22) and data on clinical results from patients with breast cancer treated with CDK4/6is are lacking. Thus, the present study assessed the value of miRNAs in patients with ABC treated with CDK4/6is.

Materials and methods

Study design. Eligible patients were those treated for ABC with CDK4/6is between June and August 2022. A total of 80 female patients (median age, 59.5 year; age range, 33-84 years) were followed-up at the Breast Cancer Centre at the Maria Skłodowska-Curie National Research Institute of Oncology (Gliwice, Poland). Patients were monitored for ≥7 months after miRNA assessment until the data cut-off in March 2023.

During CDK4/6i treatment, patients visited Maria Skłodowska-Curie National Research Institute of Oncology every 28 days for a thorough history to identify potential symptoms, physical examination and a routine blood test. A contrast-enhanced CT (Somatom Definition Edge Plus, Siemens AG; IQon Spectral CT, Philips Healthcare) was performed every 3 months. Additionally, 18-fluorodeoxyglucose positron emission tomography (PET)/CT or magnetic resonance imaging was performed at the discretion of the treating physician.

Tumor response was assessed according to RECIST 1.1 criteria and determined to be complete response (CR), partial response (PR), stable disease (SD) or progressive disease (PD) (26). CR, PR and SD comprised clinical benefit. OS was defined as time from diagnosis of the metastatic disease to time of death or last follow-up. PFS was measured from the CDK4/6i commencement date to occurrence of PD or death.

The primary objective of the present study was to assess whether levels of circulating miRNAs differed between patients with disease progression and patients deriving clinical benefit (defined as SD, PR or CR) from CDK4/6i treatment. The secondary objective was to assess whether levels of circulating miRNAs differed between patients at the beginning of the CDK4/6i treatment and patients exhibiting disease progression.

Blood processing and serum isolation. All clinical samples were obtained from subjects who provided written informed consent. Blood (10 ml) was collected on day 1 of CDK4/6i treatment in collection tubes that maintained the draw-time concentration of cell-free RNA (cfRNA; RNA Complete BCT[®] CE; Streck LLC).

All laboratory procedures were performed in the Department of Clinical and Molecular Genetic at Maria Skłodowska-Curie National Research Institute of Oncology. Blood was processed for plasma isolation within 1 h of collection. Blood was centrifuged in cfRNA collection tubes at 1,800 x g at room temperature in an Eppendorf 5810R for 15 min. Plasma was transferred to a fresh tube and centrifuged

at 2,800 x g at room temperature for 15 min. Plasma was aliquoted, with inversion to mix each aliquot, and stored at -80°C.

miRNA isolation and measurement of in plasma using reverse transcription (RT)-quantitative (q)PCR. TaqMan™ miRNA ABC Purification Kit-Human Panel A (cat. no. 4473087; Thermo Fisher Scientific, Inc.) was used for miRNA isolation from plasma samples. TaqMan Advanced miRNA cDNA Synthesis kit (cat. no. A28007; Thermo Fisher Scientific, Inc.) was used for preparing the cDNA templates from miRNA. The kit enables analysis of samples that are limited in quantity, including plasma. Mature miRNAs from total RNA were modified by extending the 3' end of the mature transcript through poly(A) addition; the 5' end was lengthened by the 5' end adaptor ligation. The modified miRNAs underwent universal RT, followed by amplification to increase the amount of cDNA uniformly for all miRNAs (miR-Amp reaction). The RT reaction and cDNA amplification were carried out using Veriti Dx 96-well Fast Thermal Cycler (Thermo Fisher Scientific, Inc.). RT was performed using a double-stage program, as follows: 15 min at 42°C and 5 min at 85°C. cDNA amplification was carried out using the following conditions: 5 min at 95°C, followed by 14 cycles of 3 sec at 95°C and 30 sec at 60°C, and a final step for 10 min at 99°C.

Analysis of miRNA expression was performed using the TaqMan Fast Advanced Master Mix for qPCR and Custom TaqMan Array Advanced MicroRNA Cards-a pre-formulated primer and probe set (cat. nos. 4444963 and A34722, respectively; Thermo Fisher Scientific, Inc.). The assay can detect and quantify mature form of the miRNA from 2 µl total RNA from serum or plasma. Based on the current literature (12,13,16-25,27-31), the following miRs were chosen: let-7a and miR-9, -17, -19a, -20a, -21, -29b, -29c, -34a, -106b, -122, -124, -126, -128, -145, -193b, -200a, -200b, -200c, -222, -223, -326 and -451.

qPCR was performed using the QuantStudio™ 12K Flex Real-Time PCR System (Thermo Fisher Scientific, Inc.) with a TaqMan Array Micro Fluidic Thermal Cycling Block (Thermo Fisher Scientific, Inc.) with the following conditions: 20 sec at 95°C, followed by 40 cycles of 1 sec at 95°C and 20 sec at 60°C. Nucleic acid-free pipette tips were used to handle all reagents. All procedures were performed according to the manufacturer's protocol.

The Pfaffl method was used to calculate relative gene expression values (32). These values were divided by the normalization ratio, prepared with the GeNorm VBA applet for Microsoft Excel (version 3.4) (33). miR-16-5p, miR-222-3p and miR-21-5p were found the most stably expressed miRNAs across all samples (34) and were used as the control/house-keeping genes (35-39).

Statistical analysis. Continuous data are presented as median and interquartile range (IQR, 25-75%). Wilcoxon rank-sum test was performed to compare the expression levels of miRNAs (separately for each miRNA; the number of miRNAs tested was 23) between groups [patients with PD vs. patients with clinical benefit (SD, PR or CR) and patients with PD vs. patients at the beginning of CDK4/6i treatment separately]. P-values were adjusted using Benjamini-Hochberg correction. OS and

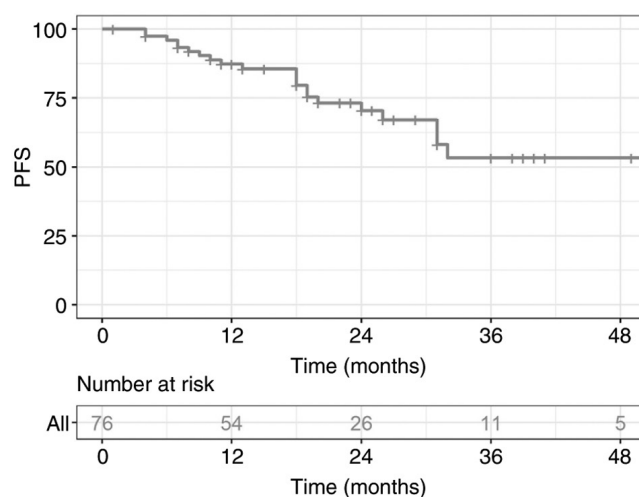


Figure 1. PFS in the studied population. PFS, progression-free survival.

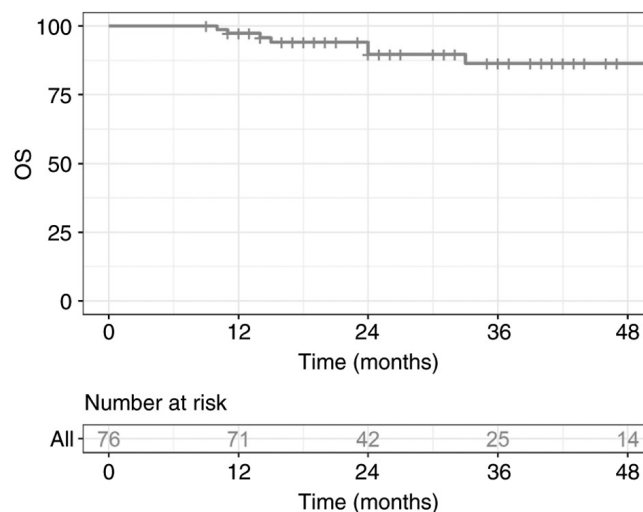


Figure 2. OS in the studied population. OS, overall survival.

PFS were estimated using the Kaplan-Meier method and 95% confidence intervals (CIs) for the survival curves were calculated. Spearman's correlation coefficient was used to assess correlation between miRNAs. The interpretation of correlation coefficient was as follows: Negligible, $0.0 \leq r < 0.1$; weak, $0.1 \leq r \leq 0.39$; moderate, $0.4 \leq r \leq 0.69$; strong, $0.7 \leq r \leq 0.89$ and very strong correlation, $0.9 \leq r \leq 1$ (40). $P < 0.05$ was considered to indicate a statistically significant difference. All computational analysis was performed in R Environment for Statistical Computing version 4.0.1 'See Things Now' (R Foundation for Statistical Computing; r-project.org).

Results

Patient characteristics. A total of 80 consecutive patients with ABC treated with CDK4/6is in Maria Skłodowska-Curie National Research Institute of Oncology were assessed. The median age was 59.5 (IQR, 50-68 years) and 19 (24%) patients were <50 years old. *De novo* disease was present in 38 patients (47.5%), whereas 42 patients (52.5%) had recurrent disease. Most

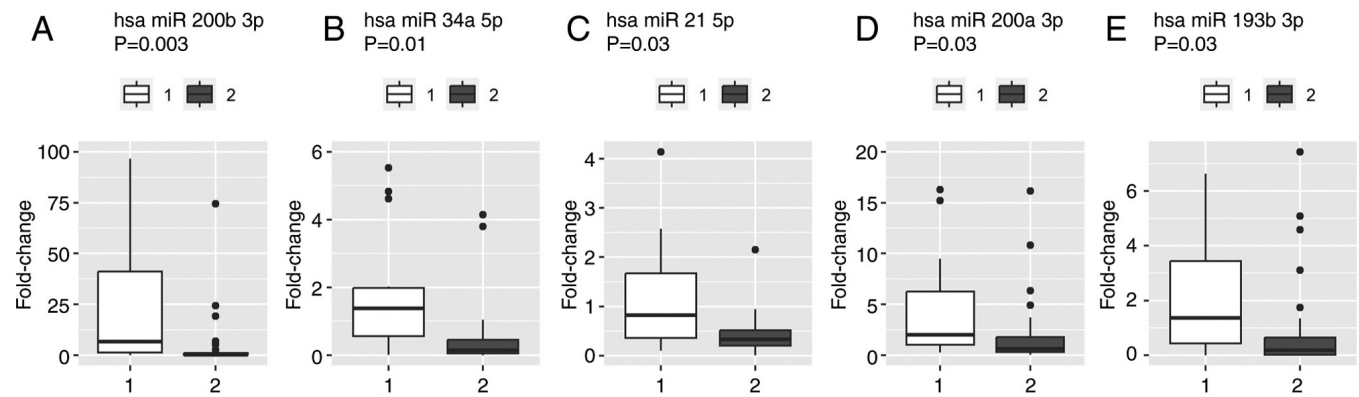


Figure 3. miRNA expression in patients with progressive disease (group 1) compared with patients with clinical benefit (stable disease + partial response + complete response; group 2). Statistically significant miR expression levels of (A) miR-200b, (B) miR-34a, (C) miR-21, (D) miR-200a and (E) miR-193b. miR, microRNA.

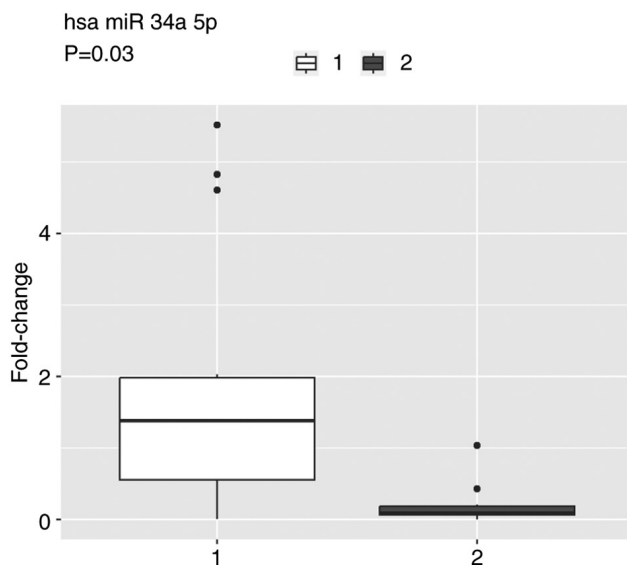


Figure 4. miR-34a expression in patients with progressive disease (group 1) compared with patients at beginning of CDK4/6 inhibitor treatment (group 2). miR, microRNA.

patients were treated in the first-line setting ($n=63$; 78.8%) and the rest were treated in the second-line. The majority of patients had bone metastasis ($n=73$; 91.3%), including 28 patients (35%) with bone-only disease, whereas seven patients (9%) had visceral metastases only. A total of 39 patients (49%) previously received chemotherapy, including 11 patients (14%) treated within 1 year of CDK4/6i commencement. Ribociclib was administered to 39 patients (49%), 22 were treated with palbociclib (27%) and 19 with abemaciclib (24%), and 58 patients (73%) received letrozole and 22 (27%) fulvestrant as an endocrine compound. A total of 41 patients (51%) had an Eastern Cooperative Oncology Group (ECOG) performance status (41) of 0, 27 had ECOG 1 (34%) and 12 had ECOG 2 (15%). Elevated cancer antigen (CA)15-3 was found in 50 patients (62.5%, median 47.0 U/ml, IQR 23.1-169.2; reference range <31.3 U/ml) (42). Blood was collected from 11 patients at the beginning of CDK4/6i treatment (9 patients on day 1 of cycle 1 and 2 patients on day 1 of cycle 2). Furthermore, blood was collected from 23 patients between cycles 3 and 10, 22 patients between cycles 11 and 20,

12 patients between cycles 21 and 30, and 12 patients beyond 30 cycles of CDK4/6i treatment. The median follow-up was 20.7 months (IQR, 12.1-29.3 months).

Treatment efficacy. At time of sampling, 14 patients were diagnosed with PD, whereas 55 patients exhibited clinical benefit from CDK4/6i treatment (including 34 patients with SD, 20 with PR and 1 with CR). Patients diagnosed with disease progression were in the following cycles of CDK4/6i treatment: Cycle 3 ($n=1$), 7 ($n=2$), 8 ($n=1$), 10 ($n=1$), 11 ($n=1$), 18 ($n=2$), 20 ($n=1$), 21 ($n=2$), 24 ($n=1$), 29 ($n=1$) and 32 ($n=1$). A total of 11 patients were at the beginning of the treatment before the first radiological response evaluation, 46 patients had baseline PET/CT and three patients achieved a complete metabolic response. The median PFS (Fig. 1) was not reached, whereas the 24-month PFS was 70.4% (95% CI, 59-84). The median OS (Fig. 2) was also not reached and the 36-month OS was 86.4% (95% CI, 76.9-97).

miRNA expression analysis. miRNA expression was measured in 76 patients (in 4 patients miRNA expression was not found), including 14 patients with PD, 51 with clinical benefit and 11 at the beginning of CDK4/6i treatment. Patients with disease progression had significantly higher levels of miR-21 ($P=0.027$), miR-34a ($P=0.011$), miR-193b ($P=0.032$), miR-200a ($P=0.027$) and miR-200b ($P=0.003$) compared with patients with clinical benefit of CDK4/6i treatment (Fig. 3). Statistically significant differences were not demonstrated for the remaining miRNAs.

miRNA expression in patients with disease progression differed from patients at the beginning of CDK4/6i treatment. Significantly higher miR-34a expression was observed in patients with PD ($P=0.031$; Fig. 4) than in patients at the beginning of treatment; however, statistically significant differences were not demonstrated for other miRNAs assessed. Moreover, significantly higher miR-122 ($P=0.070$) and miR-193b ($P=0.070$) expression was observed in patients with PD compared with the patients who were beginning treatment, whereas expression of miR-17 and miR-20a was significantly lower ($P=0.070$ for both) (data not shown).

Correlations between miRs are presented in Fig. 5. A significant, strong positive correlation was observed between miR-21 and miR-222 ($r=0.72$; $P<0.001$). A significant,

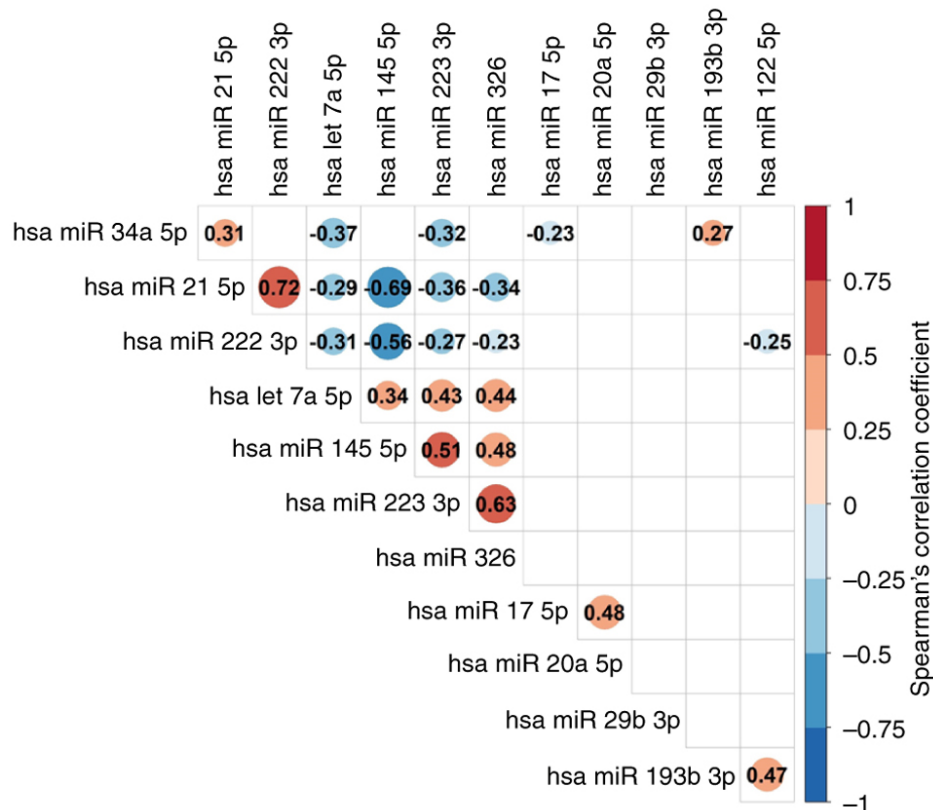


Figure 5. Correlation between miRs. A blank space indicates an insignificant correlation ($P \geq 0.05$). miR, microRNA.

moderate positive correlation was observed between miR-193b and miR-122 ($r=0.47$; $P<0.001$), miR-326 and miR-223 ($r=0.63$; $P<0.001$), let-7a and miR-223 ($r=0.43$; $P<0.001$), miR-145 and miR-223 ($r=0.51$; $P<0.001$), miR-326 and let-7a ($r=0.44$; $P<0.001$), miR-326 and miR-145 ($r=0.48$; $P<0.001$), miR-20a and miR-17 ($r=0.48$; $P<0.001$), let-7a and miR-223 ($r=0.43$; $P<0.001$). A significant, moderate negative correlation was observed between miR-145 and miR-21 ($r=-0.69$; $P<0.001$), and miR-145 and miR-222 ($r=-0.56$; $P<0.001$).

Levels of CA15-3 were also elevated in patients with PD compared with patients exhibiting the clinical benefit of CDK4/6i treatment ($P<0.001$) (data not shown).

Discussion

The present study demonstrated the role of circulating miRNAs in patients with ABC treated with CDK4/6is. Biomarkers may provide insight into the prognosis of patients with ABC and predict the treatment response. Liquid biopsy with blood-derived biomarkers is an appealing substitute for tissue biomarkers, due to the non-invasive nature of this type of biopsy, particularly in monitoring response to treatment. Currently, serum biomarkers are less well-established than imaging in managing treatment of patients with ABC. In the plethora of biomarkers retrievable from liquid biopsy, miRNAs are promising predictive and prognostic tools (43). miRNAs are key regulators of breast cancer pathogenesis, progression and response to therapy. Furthermore, emerging preclinical data have confirmed the role of miRNAs as potential predictors of response to CDK4/6i treatment (12). The stability of

miRNAs in plasma is an important prerequisite for their use as biomarkers (44). The present study assessed the role of a rationally selected subset of miRNA in patients with ABC treated with CDK4/6i.

In the present study, miRNAs conferring resistance to treatment with CDK4/6is were elevated in plasma derived from patients with PD. miR-21 is a well-known oncogene associated with protection of tumor cells from apoptosis, affecting metastasis and invasion of breast cancer (45,46). A meta-analysis of 1,629 breast cancer cases highlighted the predictive value of miR-21 expression in both breast cancer tissue and plasma samples (47). Consistent with the aforementioned studies, the present study demonstrated that miR-21 expression was elevated in patients with disease progression. In a previous study, miR-193b targeted cyclin D1 in prostate cancer and CDK4/6i inhibited proliferation of prostate cancer cell lines expressing low levels of miR-193b but did not affect the proliferation of cells with high miR-193b expression (27). Another group of miRNAs commonly dysregulated in breast cancer is the miR-200 family. miR-200a, a potential 'cell cycle break', decreases response to CDK4/6i by decreasing CDK6 expression; low miRNA-200a expression results in a more marked response to CDK4/6i in metastatic melanoma (28). miR-200b dysregulation is involved in chemoresistance by regulating drug-associated cellular pathways (29). miR-200b is upregulated in various types of cancer and downregulated by treatment with antimetabolites, such as 5-fluorouracil (48).

The present study demonstrated that both miR-200a and miR-200b were elevated in patients with PD compared with patients responding to CDK4/6is. By contrast, the present

study did not observe a difference in expression of miR-200c. miR-200c serves an antioncogenic role in renal cell cancer by controlling cell proliferation and cell cycle progression by downregulating the G1-S regulator CDK2 (30). It also increases the sensitivity of breast cancer cells to doxorubicin (31).

Most previous studies concerning miRNAs as predictors of CDK4/6is treatment were based on cell lines, fresh frozen tissue or animal models (13,22,49). To the best of our knowledge, the present study is the first to demonstrate the value of circulating miRNAs in patients with HR⁺/HER2⁺ ABC treated with CDK4/6is. Nonetheless, in several studies concerning chemotherapy-based treatment, circulating miRNAs have been reported to exhibit prognostic and predictive value, suggesting distinctive signatures associated with poor clinical outcomes (50,51). Most of the aforementioned studies focused on the triple-negative breast cancer subtype (52,53). In the HER2⁺ breast cancer subtype, circulating miRNAs are putative biomarkers of response to chemotherapy and anti-HER2 treatment (25). Circulating miR-451a, miR-16, miR-17 and miR-940 exhibit predictive value for response to trastuzumab and serve as useful biomarkers for personalized therapy (25). To the best of our knowledge, however, evidence of using miRNA as a predictor of response to CDK4/6is is lacking. miR-940 was not included in the present analysis as it is not available in the TaqMan miRNA ABC Purification Kit-Human Panel A used. However, the results of the present study suggested that miR-451a and miR-17 may be useful in distinguishing patients responding to CDK4/6i and those refractory to that treatment; however, following correction for multiple testing, the difference was insignificant.

CA15-3 has value in the management of metastatic disease; however, specificity remains low. CA15-3 is a carbohydrate-containing protein antigen of the transmembrane glycoprotein mucin-1, inhibiting tumor cell lysis and decreasing cell-cell interactions (43). Tampellini *et al* (54) assessed use of the kinetics of CA 15-3 in patients with metastatic breast cancer receiving anthracycline-based chemotherapy. Median time to disease progression was longer in patients with CA15-3 within a normal range than in patients with increased levels. CA15-3 elevation was reported in 50 patients (62.5%), which complemented the findings of other studies (55-57).

Increasing evidence suggests that the hepatocyte growth factor/mesenchymal-epithelial transition factor (c-MET) signaling pathway serves a key role in carcinogenesis, regulation of tumor microenvironment, metastasis and drug resistance (58,59). Aberrations in c-MET have been described among several potential mechanisms of resistance to CDK4/6is (60). Increased c-MET expression, frequently observed in HR⁺/HER2⁺ breast cancer (61,62), is associated with disease stage, progesterone receptor levels, Ki67 index and worse survival (63).

A technology based on TaqMan low-density array cards, used in the present study, is a recognized tool for circulating miRNA analysis (64). Nonetheless, the present study did not observe differences in miR-9, miR-124 and miR-126 expression. miR-126 may be involved in cell cycle regulation, particularly in the M phase, and improves the effect of ribociclib in the MCF7 breast cancer cell line (22).

One limitation of the present study is that the origin of the identified miRNAs was not verified and the association between miRNA expression with the corresponding breast cancer tissue

was not analyzed. The signature obtained by analyzing circulating miRNA levels expression matches the corresponding tumor tissue in a previous study (7), whereas another reported differences between plasma and tissue expression (65). Secondly, miRNA expression was only assessed at a single time point. Serial assessment, with blood collected at the beginning of and then during treatment, may confer additional insight into miRNA dynamics. To the best of our knowledge, the present study is the first to assess circulating miRNA in patients with ABC treated with CDK4/6is. Longitudinal studies are required to verify the impact of a selected set of miRNAs on objective response. The results of the present study provide a foundation for the design of such a trial.

Although the field of biomarker-associated studies in cancer continues to grow (66,67), only a few are considered standard of care for clinical practice. If confirmed in prospective clinical trials, the present miRNA signature may be an important non-invasive tool to determine treatment response, thus allowing timely treatment alternatives. Various techniques of assessing plasma miRNAs, including those presented in this study, are widely used in laboratories with a reasonable cost of reagents. Thus, with the combination of liquid biopsy and radiological assessment, personalized medicine could be integrated into the standard of care.

In summary, the present study suggested that plasma-based expression of miR-21, -34a, -193b, -200a and -200b effectively distinguished patients with ABC who respond to CDK4/6i treatment from patients who are resistant. However, the results require confirmation in larger prospective trials and longitudinal studies are required to verify use of miRNA in monitoring CDK4/6i treatment.

Acknowledgements

Not applicable.

Funding

The present study was supported by the National Science Centre, Poland (grant no. 2021/05/X/NZ5/00971).

Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

MK, MJ and MOW conceived the study. MK, MJ, MOW, PT and TT designed experiments. MK, AK and TT analyzed data. AK performed graphical presentation of results. MJ and MK developed the clinical data. PT and TT carried out the laboratory work. MK wrote the manuscript. MK, MJ, MOW, AK, PT and TT reviewed the manuscript. MK, MJ and MOW supervised the study. PT and TT confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was performed in accordance with the 1964 Declaration of Helsinki and with the ethical standards of the

Institutional Ethics Committee at Maria Skłodowska-Curie National Research Institute of Oncology (Gliwice, Poland), which approved the study (approval no. KB/430-05/22). All data were entered into an anonymized database following the general data protection regulation of the Maria Skłodowska-Curie National Research Institute of Oncology. All patients provided written informed consent before enrollment.

Patient consent for publication

Not applicable.

Competing interests

MK declares conference fees for Pfizer, Roche, Novartis, Teva, and Amgen; clinical trials for Roche, MSD, Novartis, Seagen, and Gilead; speaker's honoraria from Novartis, Roche, Lilly, Teva, and Amgen, Swixx Biopharma; advisory board for Novartis; all outside the submitted work. MJ declares conference fees for Gilead, Roche; clinical trials for Roche, MSD, Novartis, Seagen, and Gilead; speaker's honoraria from Novartis, Roche, Lilly, Pfizer, Teva, Exact Sciences, and Mammutome; advisory boards for Novartis and Pfizer; all outside submitted work. All other authors declare they have no competing interests.

References

- Arnold M, Morgan E, Rumgay H, Mafra A, Singh D, Laversanne M, Vignat J, Gralow JR, Cardoso F, Siesling S and Soerjomataram I: Current and future burden of breast cancer: Global statistics for 2020 and 2040. *Breast* 66: 15-23, 2022.
- Miglietta F, Bottosso M, Griguolo G, Dieci MV and Guarneri V: Erratum to 'Major advancements in metastatic breast cancer treatment: When expanding options means prolonging survival'. [ESMO Open Volume 7, Issue 2, April 2022, 100409]. *ESMO Open* 7: 100472, 2022.
- Cetin B, Wabl CA and Gumusay O: CDK4/6 inhibitors: Mechanisms of resistance and potential biomarkers of responsiveness in breast cancer. *Future Oncol* 18: 1143-1157, 2022.
- Hortobagyi GN: Ribociclib for the first-line treatment of advanced hormone receptor-positive breast cancer: A review of subgroup analyses from the MONALEESA-2 trial. *Breast Cancer Res* 20: 123, 2018.
- Eisenhauer EA, Therasse P, Bogaerts J, Schwartz LH, Sargent D, Ford R, Dancey J, Arbuck S, Gwyther S, Mooney M, *et al*: New response evaluation criteria in solid tumours: revised RECIST guideline (version 1.1). *Eur J Cancer* 45: 228-247, 2009.
- Caschera L, Lazzara A, Piergallini L, Ricci D, Tuscano B and Vanzulli A: Contrast agents in diagnostic imaging: Present and future. *Pharmacol Res* 110: 65-75, 2016.
- Matamala N, Vargas MT, González-Cámpora R, Miñambres R, Arias J, Menéndez P, Andrés-León E, Gómez-López G, Yanowsky K, Calvete-Candenas J, *et al*: Tumor microRNA expression profiling identifies circulating microRNAs for early breast cancer detection. *Clin Chem* 61: 1098-1106, 2015.
- Kim VN and Nam JW: Genomics of microRNA. *Trends Genet* 22: 165-173, 2006.
- Di Leva G and Croce CM: Roles of small RNAs in tumor formation. *Trends Mol Med* 16: 257-267, 2010.
- Croce CM: Causes and consequences of microRNA dysregulation in cancer. *Nat Rev Genet* 10: 704-714, 2009.
- Johnson SM, Grosshans H, Shingara J, Byrom M, Jarvis R, Cheng A, Labourier E, Reinert KL, Brown D and Slack FJ: RAS is regulated by the let-7 microRNA family. *Cell* 120: 635-647, 2005.
- Andrikopoulou A, Shalit A, Zografos E, Koutsoukos K, Korakiti AM, Lontos M, Dimopoulos MA and Zagouri F: MicroRNAs as potential predictors of response to CDK4/6 inhibitor treatment. *Cancers (Basel)* 13: 4114, 2021.
- Ji W, Zhang W, Wang X, Shi Y, Yang F, Xie H, Zhou W, Wang S and Guan X: c-myc regulates the sensitivity of breast cancer cells to palbociclib via c-myc/miR-29b-3p/CDK6 axis. *Cell Death Dis* 11: 760, 2020.
- Qattan A, Intabli H, Alkhayal W, Eltabache C, Tweigieri T and Amer SB: Robust expression of tumor suppressor miRNAs let-7 and miR-195 detected in plasma of Saudi female breast cancer patients. *BMC Cancer* 17: 799, 2017.
- Eichelser C, Flesch-Janys D, Chang-Claude J, Pantel K and Schwarzenbach H: Deregulated serum concentrations of circulating cell-free microRNAs miR-17, miR-34a, miR-155, and miR-373 in human breast cancer development and progression. *Clin Chem* 59: 1489-1496, 2013.
- Gary JM, Simmons JK, Xu J, Zhang S, Peat TJ, Watson N, Gamache BJ, Zhang K, Kovalchuk AL, Michalowski AM, *et al*: Hypomorphic mTOR downregulates CDK6 and Delays Thymic Pre-T LBL tumorigenesis. *Mol Cancer Ther* 19: 2221-2232, 2020.
- Rodríguez-Otero P, Román-Gómez J, Vilas-Zornoza A, José-Eneriz ES, Martín-Palanco V, Rifón J, Torres A, Calasanz MJ, Agirre X and Prosper F: Deregulation of FGFR1 and CDK6 oncogenic pathways in acute lymphoblastic leukaemia harbouring epigenetic modifications of the MIR9 family. *Br J Haematol* 155: 73-83, 2011.
- Sakai A, Saitow F, Maruyama M, Miyake N, Miyake K, Shimada T, Okada T and Suzuki H: MicroRNA cluster miR-17-92 regulates multiple functionally related voltage-gated potassium channels in chronic neuropathic pain. *Nat Commun* 8: 16079, 2017.
- Li M, Xiao A, Floyd D, Olmez I, Lee J, Godlewski J, Bronisz A, Bhat KPL, Sulman EP, Nakano I and Purow B: CDK4/6 inhibition is more active against the glioblastoma proneural subtype. *Oncotarget* 8: 55319-55331, 2017.
- Thangavel C, Boopathi E, Ertel A, Lim M, Addya S, Fortina P, Witkiewicz AK and Knudsen ES: Regulation of miR106b cluster through the RB pathway: Mechanism and functional targets. *Cell Cycle* 12: 98-111, 2013.
- Agirre X, Vilas-Zornoza A, Jiménez-Velasco A, Martín-Subero JI, Cordeu L, Gárate L, San José-Eneriz E, Abizanda G, Rodríguez-Otero P, Fortes P, *et al*: Epigenetic silencing of the tumor suppressor microRNA Hsa-miR-124a regulates CDK6 expression and confers a poor prognosis in acute lymphoblastic leukemia. *Cancer Res* 69: 4443-4453, 2009.
- Baldassari F, Zerbinati C, Galasso M, Corrà F, Minotti L, Agnoletto C, Prevati M, Croce CM and Volinia S: Screen for microRNA and drug interactions in breast cancer cell lines points to miR-126 as a modulator of CDK4/6 and PIK3CA inhibitors. *Front Genet* 9: 174, 2018.
- Citron F, Segatto I, Vinciguerra GLR, Musco L, Russo F, Mungo G, D'Andrea S, Mattevi MC, Perin T, Schiappacassi M, *et al*: Downregulation of miR-223 expression is an early event during mammary transformation and confers resistance to CDK4/6 inhibitors in luminal breast cancer. *Cancer Res* 80: 1064-1077, 2020.
- Favero A, Segatto I, Perin T and Belletti B: The many facets of miR-223 in cancer: Oncosuppressor, oncogenic driver, therapeutic target, and biomarker of response. *Wiley Interdiscip Rev RNA* 12: e1659, 2021.
- Li H, Liu J, Chen J, Wang H, Yang L, Chen F, Fan S, Wang J, Shao B, Yin D, *et al*: A serum microRNA signature predicts trastuzumab benefit in HER2-positive metastatic breast cancer patients. *Nat Commun* 9: 1614, 2018.
- Eisenhauer EA, Therasse P, Bogaerts J, Schwartz LH, Sargent D, Ford R, Dancey J, Arbuck S, Gwyther S, Mooney M, *et al*: New response evaluation criteria in solid tumours: Revised RECIST guideline (version 1.1). *Eur J Cancer* 45: 228-247, 2009.
- Kaukonen KM, Rauhala HE, Scaravilli M, Latonen L, Annala M, Vessella RL, Nykter M, Tammela TL and Visakorpi T: Epigenetically altered miR-193b targets cyclin D1 in prostate cancer. *Cancer Med* 4: 1417-1425, 2015.
- Bustos MA, Ono S, Marzese DM, Oyama T, Iida Y, Cheung G, Nelson N, Hsu SC, Yu Q and Hoon DSB: MiR-200a Regulates CDK4/6 Inhibitor Effect by Targeting CDK6 in Metastatic Melanoma. *J Invest Dermatol* 137: 1955-1964, 2017.
- Jayaraj R, Nayagam SG, Kar A, Sathyakumar S, Mohammed H, Smiti M, Sabarimurugan S, Kumarasamy C, Priyadharshini T, Gothandam KM, *et al*: Clinical therapeutic relationship between drug-resistance specific mirna expressions, chemotherapeutic resistance, and sensitivity in breast cancer: A systematic review and Meta-Analysis. *Cells* 8: 1250, 2019.

30. Wang X, Chen X, Han W, Ruan A, Chen L, Wang R, Xu Z, Xiao P, Lu X, Zhao Y, *et al*: miR-200c Targets CDK2 and suppresses tumorigenesis in renal cell carcinoma. *Mol Cancer Res* 13: 1567-1577, 2015.
31. Safaei S, Amini M, Najjary S, Mokhtarzadeh A, Bolandi N, Saeedi H, Alizadeh N, Javadrashid D and Baradaran B: miR-200c increases the sensitivity of breast cancer cells to Doxorubicin through downregulating MDR1 gene. *Exp Mol Pathol* 125: 104753, 2022.
32. Pfaffl MW: A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* 29: e45, 2001.
33. Hildyard JCW and Wells DJ: Identification and validation of quantitative PCR reference genes suitable for normalizing expression in normal and dystrophic cell culture models of myogenesis. *PLoS Curr* 6: ecurrents.md.faafdde4bea8df4aa7d06cd5553119a6, 2014.
34. Rinnerthaler G, Hackl H, Gampenrieder SP, Hamacher F, Hufnagl C, Hauser-Kronberger C, Zehentmayr F, Fastner G, Sedlmayer F, Mlineritsch B and Greil R: miR-16-5p Is a Stably-Expressed housekeeping MicroRNA in breast cancer tissues from primary tumors and from metastatic sites. *Int J Mol Sci* 17: 156, 2016.
35. Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A and Speleman F: Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol* 3: RESEARCH0034, 2002.
36. Pfaffl MW, Tichopad A, Prgomet C and Neuvians TP: Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper-Excel-based tool using pair-wise correlations. *Biotechnol Lett* 26: 509-515, 2004.
37. Radonić A, Thulke S, Mackay IM, Landt O, Siegert W and Nitsche A: Guideline to reference gene selection for quantitative real-time PCR. *Biochem Biophys Res Commun* 313: 856-862, 2004.
38. Andersen CL, Jensen JL and Ørntoft TF: Normalization of real-time quantitative reverse transcription-PCR data: A model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. *Cancer Res* 64: 5245-5250, 2004.
39. Dheda K, Huggett JF, Bustin SA, Johnson MA, Rook G and Zumla A: Validation of housekeeping genes for normalizing RNA expression in real-time PCR. *Biotechniques* 37: 112-119, 2004.
40. Schober P and Schwarte LA: Correlation coefficients: Appropriate use and interpretation. *Anesth Analg* 126: 1763-1768, 2018.
41. Oken MM, Creech RH, Tormey DC, Horton J, Davis TE, McFadden ET and Carbone PP: Toxicity and response criteria of the Eastern Cooperative Oncology Group. *Am J Clin Oncol* 5: 649-656, 1982.
42. Duffy MJ: Biochemical markers in breast cancer: Which ones are clinically useful? *Clin Biochem* 34: 347-352, 2001.
43. Seale KN and Tkaczuk KHR: Circulating biomarkers in breast cancer. *Clin Breast Cancer* 22: e319-e331, 2022.
44. Mitchell PS, Parkin RK, Kroh EM, Fritz BR, Wyman SK, Pogosova-Agadjanyan EL, Peterson A, Noteboom J, O'Brian KC, Allen A, *et al*: Circulating microRNAs as stable blood-based markers for cancer detection. *Proc Natl Acad Sci USA* 105: 10513-10518, 2008.
45. Song B, Wang C, Liu J, Wang X, Lv L, Wei L, Xie L, Zheng Y and Song X: MicroRNA-21 regulates breast cancer invasion partly by targeting tissue inhibitor of metalloproteinase 3 expression. *J Exp Clin Cancer Res* 29: 29, 2010.
46. Wang H, Tan Z, Hu H, Liu H, Wu T, Zheng C, Wang X, Luo Z, Wang J, Liu S, *et al*: microRNA-21 promotes breast cancer proliferation and metastasis by targeting LZTFL1. *BMC Cancer* 19: 738, 2019.
47. Jinling W, Sijing S, Jie Z and Guinian W: Prognostic value of circulating microRNA-21 for breast cancer: A systematic review and meta-analysis. *Artif Cells Nanomed Biotechnol* 45: 1-6, 2017.
48. Rossi L, Bonmassar E and Faraoni I: Modification of miR gene expression pattern in human colon cancer cells following exposure to 5-fluorouracil in vitro. *Pharmacol Res* 56: 248-253, 2007.
49. Yu Y, Liao H, Xie R, Zhang Y, Zheng R, Chen J and Zhang B: Overexpression of miRNA-3613-3p enhances the sensitivity of triple negative breast cancer to CDK4/6 inhibitor palbociclib. *Front Oncol* 10: 590813, 2020.
50. van Zweeken AA, Opperman RCM, Honeywell RJ, Peters GJ, Verheul HMW, van der Vliet HJ and Poel D: The prognostic impact of circulating miRNAs in patients with advanced esophagogastric cancer during palliative chemotherapy. *Cancer Treat Res Commun* 27: 100371, 2021.
51. Xiao W, Zhong Y, Wu L, Yang D, Ye S and Zhang M: Prognostic value of microRNAs in lung cancer: A systematic review and meta-analysis. *Mol Clin Oncol* 10: 67-77, 2019.
52. Sahlberg KK, Bottai G, Naume B, Burwinkel B, Calin GA, Børresen-Dale AL and Santarpia L: A serum MicroRNA signature predicts tumor relapse and survival in triple-negative breast cancer patients. *Clin Cancer Res* 21: 1207-1214, 2015.
53. Piña-Sánchez P, Valdez-Salazar HA and Ruiz-Tachiquín ME: Circulating microRNAs and their role in the immune response in triple-negative breast cancer. *Oncol Lett* 20: 224, 2020.
54. Tampellini M, Berruti A, Bitossi R, Gorzegno G, Alabiso I, Bottini A, Farris A, Donadio M, Sarobba MG, Manzin E, *et al*: Prognostic significance of changes in CA 15-3 serum levels during chemotherapy in metastatic breast cancer patients. *Breast Cancer Res Treat* 98: 241-248, 2006.
55. De Cock L, Heylen J, Wildiers A, Punie K, Smeets A, Weltens C, Neven P, Billen J, Laenen A and Wildiers H: Detection of secondary metastatic breast cancer by measurement of plasma CA 15.3. *ESMO Open* 6: 100203, 2021.
56. Yerushalmi R, Tyldesley S, Kennecke H, Speers C, Woods R, Knight B and Gelmon KA: Tumor markers in metastatic breast cancer subtypes: Frequency of elevation and correlation with outcome. *Ann Oncol* 23: 338-345, 2012.
57. Yang Y, Zhang H, Zhang M, Meng Q, Cai L and Zhang Q: Elevation of serum CEA and CA15-3 levels during antitumor therapy predicts poor therapeutic response in advanced breast cancer patients. *Oncol Lett* 14: 7549-7556, 2017.
58. Fu J, Su X, Li Z, Deng L, Liu X, Feng X and Peng J: HGF/c-MET pathway in cancer: From molecular characterization to clinical evidence. *Oncogene* 40: 4625-4651, 2021.
59. Faiella A, Riccardi F, Carteni G, Chiurazzi M and Onofrio L: The Emerging Role of c-met in carcinogenesis and clinical implications as a possible therapeutic target. *J Oncol* 2022: 5179182, 2022.
60. Zhou FH, Downton T, Freeland A, Hurwitz J, Caldon CE and Lim E: CDK4/6 inhibitor resistance in estrogen receptor positive breast cancer, a 2023 perspective. *Front Cell Dev Biol* 11: 1148792, 2023.
61. Yan S, Jiao X, Zou H and Li K: Prognostic significance of c-Met in breast cancer: A meta-analysis of 6010 cases. *Diagn Pathol* 10: 62, 2015.
62. Ho-Yen CM, Jones JL and Kermorgant S: The clinical and functional significance of c-Met in breast cancer: A review. *Breast Cancer Res* 17: 52, 2015.
63. Iovino F, Diana A, Carlino F, Ferraraccio F, Antoniol G, Fisone F, Perrone A, Zito Marino F, Panarese I, Tathode MS, *et al*: Expression of c-MET in estrogen receptor positive and HER2 negative resected breast cancer correlated with a poor prognosis. *J Clin Med* 11: 6987, 2022.
64. Zearo S, Kim E, Zhu Y, Zhao JT, Sidhu SB, Robinson BG and Soon PSh: MicroRNA-484 is more highly expressed in serum of early breast cancer patients compared to healthy volunteers. *BMC Cancer* 14: 200, 2014.
65. Li M, Zou X, Xia T, Wang T, Liu P, Zhou X, Wang S and Zhu W: A five-miRNA panel in plasma was identified for breast cancer diagnosis. *Cancer Med* 8: 7006-7017, 2019.
66. Sarhadi VK and Armengol G: Molecular biomarkers in cancer. *Biomolecules* 12: 1021, 2022.
67. Khan H, Shah MR, Barek J and Malik MI: Cancer biomarkers and their biosensors: A comprehensive review. *TrAC Trends Anal Chem* 158: 116813, 2023.



Copyright © 2024 Kubeczko *et al*. This work is licensed under a Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International (CC BY-NC-ND 4.0) License.