MiRNAs-181a/b as Predictive biomarkers for olaparib sensitivity in triple-negative breast cancer cells.

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ABSTRACT

Background: spotting the scope on triple-negative breast cancer (TNBC) as the most aggressive type of breast cancer, with no targeted therapeutic options. TNBC is often characterized by having defects in DNA repair due to defects in BRCA making this cancer a rational target for the synthetic lethality of olaparib, as an inhibitory target agent of the alternative DNA repair pathway (poly ADP-ribose polymerase "PARP" inhibitor). **Objectives**: the present study aims to evaluate the value of miRNAs-181a/b as potential biomarkers in predicting the response of TNBC to olaparib. **Methods:** anti-miRNAs-181a/b was transfected into MDA-MB-231 cell line using HiPerFect transfection reagent, the transfected and untransfected cells were subjected to olaparib. The effect of miRNAs-181a/b on MDA-MB-231 treated cells with olaparib was evaluated through the detection of essential proteins involved in apoptosis and cell proliferation including Caspase-8, Bcl-2, and Ki-67. Further, the expression level of ataxia telangiectasia mutated (ATM) was determined as a functional target of miRNAs-181a/b. **Results:** a significant decrease in Caspase-8 activity, and Bcl-2, but a significant increase in cell survival, cell proliferation, and ATM protein were observed upon suppression of miRNAs-181a/b by their inhibitors followed by treatment with olaparib for TNBC cell line (MDA-MB-231 cells). **Conclusions:** our data confirmed that miRNA-181a and miRNA-181b play a critical role for detecting the sensitivity of TNBC cells to olaparib. As well as miRNAs-181a/b could be used as a potentially predictive biomarkers for response to olaparib.

INTRODUCTION:

Breast cancer (BC) is now one of the world`s most common neoplasm among women and the second leading reason of cancer-related death, after lung cancer (1.67 million deaths in 2012) according to the last report of the International Agency of Research on Cancer [1] . In Egypt, BC is estimated to be the most common cancer among females accounting for 38.8% of total cancer cases [2]. It is also the leading cause of cancer related mortality accounting for 29.1% of their total with 6,546 deaths. The incidence to mortality ratio is poor (1.9:1) [2]**.**

Triple-negative breast cancer (TNBC) is a heterogeneous subtype of BC in which the three receptors, estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor (HER2)

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are negatively expressed. Therefore, there is a gap in TNBC response to endocrine therapy to bridge this gap, several studies based on analyzing the TNBC expressed genes have been done to open the way for identifying novel targeted agents for TNBC as well as finding biomarkers that could be helpful in predicting sensitivity of TNBC to these agents [4].

Olaparib (poly ADP-ribose polymerase "PARP" inhibitor) has identified as a new targeted therapy inducing synthetic lethality in BRCA mutated TNBC via inhibition of PARP, with maintaining minimal normal tissue toxicity [5]. TNBC carrying mutations in BRCA genes appear to be more dependent on PARP for survival as a backup DNA repair mechanism, but the synthetic inhibition of PARP by olaparib leads to lethal double strand breaks during tumor cell replication [6].

Classes of non-coding RNAs are known as MicroRNAs [7], that is micro in their size (21-22 nucleotides) but macro in their function by influencing the evolution and stability of much mRNAs to make fine-scale adjustments to the protein synthesis tune from thousands of genes [8]. MicroRNA-181a and MicroRNAs-181b are members in the miRNA-181 family, which have been shown to be up-regulated in TNBC [9] and involved in many pathways directing cells toward angiogenesis [10]. It is likely, therefore, that they can also modulate sensitivity and resistance to anticancer drugs in substantial ways.

The main objective of the present work was to evaluate the value of miRNAs-181a/b as potential biomarkers in predicting the response of TNBC cell line (MDA-MB-231) to olaparib after transfection with their inhibitors. The level of apoptotic and cell proliferation markers was measured (caspase-8, Bcl-2, and Ki-67) in addition to the level of ATM protein.

MATERIALS AND METHODS:

Cell line and culture conditions

The MDA-MB-231 cell line, known as a wild-type for BRCA human triple-negative breast cancer cell line, was obtained from Vacsera (Dokki, Giza, Egypt) and processed in National Cancer Institute, Cairo University. The MDA-MB-231 cells were cultured in DMEM medium (Sigma, St Louis, MO, USA) containing 10% fetal bovine serum along with 100 mg/ml streptomycin and 100 units/ml penicillin G (Sigma, St Louis, MO, USA). Cells were incubated in a humidified atmosphere with 5% (v/v) CO_2 at 37°C. For drug treatment, 10mg of olaparib (LKT Laboratories, USA) was dissolved in 1ml DMSO and stored at -4° C upon usage.

Anti-tumor activity assay of olaparib on MDA-MB-321 cells

According to **Vichai and Kirtikara** protocol [11], Sulphorhodamine-B (SRB) assay was done to evaluate the inhibitory effect of olaparib on the growth of MDA-MB-231 cells. Briefly, the 70% confluence cells were harvested from the dish with 0.05% trypsin/EDTA solution and seeded at law density in the 96-well cultured plate (5×10^3 cells/well). Following overnight adherence, Cells were subsequently incubated with DMEM media alone (serve as a control) or with different concentrations of olaparib (5 μg/ml, 10 μg/ml, 20 μg/ml, 40 μg/ml and 80 μg/ml) in triplicate to induce cytotoxicity. 48 hours post olaparib treatment the cell monolayers were fixed with 10% (W/V) trichloroacetic acid, stained with SRB for 30 min, then dissolved in 10 mM Tris base solution, and the absorbance of all wells (control and treated wells) was measured at 570 nm on an ELISA microplate reader for the assessment of olaparib IC_{50}, and EC_{50}.

Cell transfection with anti-miRNAs-181a/b

Synthetic Anti-has-miRNA-181a and Anti-has-miRNA-181b were obtained from (Qiagen, Germany). MDA-MB-231 cells were transfected with 5 nM

antagomir of miRNA-181a or miRNA-181b that bind specifically to and inhibit endogenous miRNA-181a or miRNA-181b, respectively using HiPerFect transfection reagent (Qiagen) according to the following protocol; Cells were seeded and allowed to adhere overnight, and then each antagomir was mixed with HiPerFect transfection reagent in the desired volume of serum free media for 30 minutes to allow the formation of transfection complex, which was added to the target cells for 24 h incubation period [12].

Real-time PCR (qRT-PCR) of miRNAs-181a/b

The expression of miRNAs-181a/b before and after transcription was quantified to ensure the effectiveness of the transfection process. Total RNA including miRNAs isolated from cells were reversed transcribed with miScript II reverse transcriptase kit using a universal primer as described in the manufacturer's instructions. The expression of miRNAs-181a/b was analyzed using SYPER Green qRT-PCR kit (Qiagen), according to the manufacturer's instructions. The human miRNA-U6 was used as endogenous control. All the reactions were performed in triplicate on ViiA™ 7 Real-Time PCR (Applied Biosystem, USA). The relative miRNAs-181a/b expression levels were quantified by using the $2^{-\Delta\Delta Ct}$ method [13].

Cell viability by trypan blue exclusion assay

MDA-MB-231 cells were plated into 6-well plate overnight, they were transfected with Anti-miRNAs-181a/b for 24 h incubation period, and then they were incubated with or without 100μM olaparib for 48 h, the vehicle control contained only media. The given MDA-MB-231 cell population of different treated groups were harvested, stained with trypan blue and then subjected to hemocytometer for microscopic examination and enumeration of live (unstained) and dead (blue) cells [14].

Analysis of caspase-8, Bcl-2, Ki-67 and ATM protein level

MDA-MB-231 cells were allowed for seeding overnight, they were transfected with Anti-miRNAs-181a/b for 24 h incubation period, and then they were incubated with or without 100μM olaparib for 48 h. The cells were harvested by trypsin and lysed by freezing in liquid nitrogen and then thawing with gentle mixing. Using enzyme-linked immunosorbent assay according to the manufacturer instructions of ELISA kit (Biomatik, USA). The reaction product was measured at 450 nm [15].

Statistical analysis

All statistical analyses were performed using GraphPad prism software, version 5. Data obtained from three or more individual experiments were expressed as mean ± SD. Data were analyzed by One-Way ANOVA and Tukey post Hoc tests. P-values less than 0.05 were considered as statistically significant [16].

RESULTS:

Olaparib Anti-tumor activity (determining I.C.$_{50}$ and E.C.$_{50}$ in olaparib treated MDA-MB-231 cells).

To determine the 50 inhibition concentration value (I.C.$_{50}$) of olaparib on MDA-MB-231 cells, in the present study, different concentrations were used starting from 5 μg/mL ending with 80 μg/mL for 48 hours incubation period using SRP technique. Our data showed decreasing in the cellular proliferation in a concentration-dependent manner as a result of olaparib treatment, and the I.C.$_{50}$ value was 43.5μg/ml. As well as, the effective concentration (E.C.$_{50}$) of olaparib on 50% of the MDA-MB-231 cellular population was calculated using different concentrations starting from 5 μg/mL ending with 43.5 μg/mL, and its value was 37.4μg/ml, **(figure 1)**.

The expression level of miRNAs-181a/b in MDA-MB-231 cells transfected with their inhibitors (Anti-miRNAs-181a/b).

The expression level of miRNA-181a and miRNA-181b were analyzed using qRT-PCR assay before and after

transfection of their inhibitors in MDA-MB-231 cells. Significant down-regulation was observed in both miRNAs analyzed, where the fold changes recorded were 0.07 for miR-181a and 0.02 for miR-181b compared to control (un-transfected) cells ($p<0.05$).

Cell viability assay for the transfected and un-transfected MBA-MB-231 cells.

Optimization of the transfection assay with Anti-miRNAs-181a/b were done through different group of cells compared to the control un-transfected cells (100%), as shown in (figure 2), HiPerFect transfection reagent treated cells (97.2%), Anti-miRNA-181a transfected cells (98.2%), and Anti-miRNA-181b transfected cells (97.9%), ensuring the slight effect of the reagent used in transfection process on cell viability.

The survival analysis of transfected and un-transfected MBA-MB-231 cells during their period of treatment with olaparib was analyzed, where the transfected cells with Anti-miRNA-181a or Anti-miRNA-181b followed by treatment with olaparib (37.4 µg/ml) show a good survival (72%) and (77%), respectively. However, cells treated with olaparib alone showed the lowest survival rate (9%) as described in **(figure 2)**.

Effect of olaparib on the enzymatic activity of caspase-8.

The enzymatic activity of caspase-8 was measured in cells treated with anti-miRNAs-181a/b and (37.4 µg/ml) olaparib using ELISA technique. Caspase-8 activity was significantly increased in olaparib treated cells compared to untreated cells, where the activity reached 25 U/L compared to control untreated cells ($P < 0.05$). Whereas; it`s activity level was dramatically decreased after transfection with either anti-miRNA-181a or anti-miRNA-181b (it was 6.29 U/L and 7.85 U/L, respectively) indicating the synergistic effect of miRNAs-181a/b and olaparib, **(Figure 3)**.

The effect of olaparib on Bcl-2 protein expression level.

The protein level of Bcl-2 was measured in different treated groups of cells in the current study. A significant decreasing in its level was observed in olaparib treated cells alone comparing to untreated control cells where it was 9.34 ng/ml. However, the transfected cells treated with olaparib were still expressing Bcl-2 protein more than the un-transfected olaparib treated cells where the values recorded 14.82 ng/ml and 16.5 ng/ml in cells transfected with anti-miRNA-181a and anti-miRNA-181b, respectively **(Figure 4)**.

The effect of olaparib on the expression level of Ki-67 protein.

A significant decrease in the expression of Ki-67 protein in MDA-MB-231 cells, that is expressing miRNAs-181a/b, after treatment with olaparib (37.4 µg/ml) compared to untreated control cells ($p < 0.05$). Upon targeting the cells with at anti-miRNA-181a or anti-miRNA-181b and then treated with olaparib the level of Ki-67 increased significantly to reach 14.82 ng/ml and 16.5 ng/ml, respectively as shown in **(Figure 5).**

Effect of miRNA-181a and miRNA-181b on ataxia talengiastia mutated (ATM) protein level in transfected and non-transfected MBA-MB-231.

Comparing the control (un-transfected) cell that is already expressing miRNA-181a and miRNa-181b to transfected MDA-MB-231 cells with Anti-miRNA1-181a and Anti-miRNA-181b, the protein expression level of ATM was sharply increased, where the values were (471.01 pg/ml) for cells transfected with anti-miRBA-181a and (436.54 pg/ml) for anti-miRNA-181b transfected cells. This result confirms the inhibitory effect of both miRNA-181a and miRNA-181b on the mRNA expression of ATM protein, **(Figure 6)**.

DISCUSSION:

Triple-negative breast cancer (TNBC; ER, PR, and HER2-negative breast cancer) has limited therapeutic options. As a

result, conventional cytotoxic chemotherapy is the only effective systemic treatment for TNBC and there is an urgent need for new treatment approaches [17].

Poly ADP-ribose polymerase (PARP) is an enzyme plays an important role in DNA repair. Tumor cells appear to be more dependent on PARP for survival even after cytotoxic chemotherapy thus increasing the rate of tumor resistance to chemotherapy [18]. Therefore, PARP is considered a specific target in tumor treatment. Olaparib; a promising potent inhibitor of PARP by binding to PARP covalently, have an anticancer effect on many types of cancer those has defects in DNA repair cascade and also have fewer side effects than the traditional chemotherapeutic drugs [18].

Dysregulation of miRNAs in different types of cancer can occur by upstream genetic regulatory elements as well as by epigenetic modifications [19]. It has been shown that resistance to chemotherapeutic drugs in several cancers may be attributed to the alternation of miRNA expression [20]. Therefore targeting specific miRNA may serve as a new strategy to overcome drug resistance process miRNA-181a has been reported to function as a tumor oncogene in different types of cancer including breast where it plays a critical role in several cell processes including drug resistance.

Overexpression of miRNAs-181a/b was observed in breast cancer tissues and this expression was correlated positively with proliferation and aggressiveness of the tumor [21]. The substantial higher level of miRNA-181a was detected in TNBC patients [22] and is correlated was a poor distant metastasis-free survival (Niu et al., 2016). In the recent study, treatment of MDA-MB-231 cells with anti-miRNAs-181a/b inhibitors increased cell survival. A decreasing in cell survival was reported in cells with overexpression of miRNAs-181a/b and treated with PARP inhibitor which indicates that overexpression of

miRNAs-181a/b may increase the cell sensitivity to olaparib treatment.

It was shown in that deficiency in DNA damage sensor proteins such as Ataxia telangiectasia mutated (ATM) and serine/threonine-protein kinase (ATR) lead to a deficiency in homologues recombination pathway and displayed sensitivity to PARP inhibitor (olaparib) [23]. Due to different molecular factors, law level of BRCA1 expression was observed in sporadic TNBC compared to ER-positive breast cancer [24]. The majority of TNBC that are negative BRCA mutation share features with the basal-like phenotype classically associated with BRCA-related cancer that leads to homologous recombination deficiency and sensitization to PARP inhibitors [25].

For prediction of miRNAs-181a/b targeted gene using Target Scan and Miranda algorithms, ATM protein level was reported to be regulated inversely by miRNAs-181a/b [21]. Our data showed up-regulation of ATM level in MDA-MB-231 cell lines upon treatment with miRNAs-181a/b inhibitors. Another study verified that overexpression of miRNAs-181a/b leads to decreasing of ATM protein level in different cell lines derived from breast, ovary, lung and pancreas [21] All of the above data support the hypothesis that miRNAs-181a/b plays a crucial role in the regulation of ATM protein expression and has an important impact on the cellular response through dampening DNA repair process by HR that increase sensitivity to PARP inhibitor treatment.

A defect in the apoptotic pathway is considered to be one of the main mechanisms of drug resistance in cancer. The main role of miRNAs-181 a/b in regulating chemotherapeutic resistance depending on tumor types and drug investigated. It was reported that upregulation of miRNA-181a in non-small cell lung cancer cells enhances apoptosis upon treatment with cisplatin [26].

A recent study showed that induction of miRNAs-181a/b repressed apoptosis in

response to doxorubicin treatment in TNBC MDA-MB-231 cells [22] where the Bax gene (pro-apoptotic gene) was identified as the direct target of miRNA-181a [22] has a major role in mediating doxorubicin resistance. In another study, it was found that up-regulation of anti-apoptotic protein Bcl-2 with the down-regulation of miRNA-181b in resistance of gastric and lung cells [26]. In our study, inhibiting the expression of miRNAs-181a/b in treated MDA-MB-231 cells with olaparib decreased the Bcl-2 level. This decreasing has no significant difference with cells treated with olaparib alone. This phenomenon could be explained that the decreasing of Bcl-2 level and induction of apoptosis via a pathway miRNAs-181a/b is not necessary involved.

CONCLUSION:

Taken together, our data suggested that overexpression of miRNAs-181a/b in TNBC could be a biomarker for response to PARP inhibitor. Moreover, miRNAs-181a/b – ATM pathway determine the sensitivity to olaparib as PARP inhibitor treatment.

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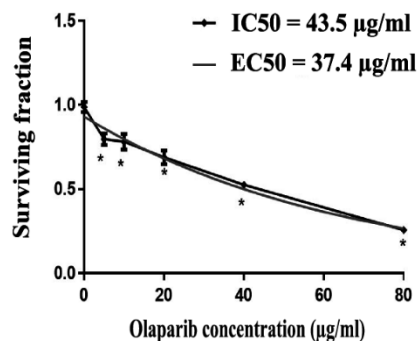
Figure (1): Surviving fraction chart of SRP assay results, I.C.50 and E.C.50 of MDA-MB-231 treated with different concentration of olaparib (5-80 μg/ml) for 48 h. * significantly different at $P < 0.05$ compared to control (untreated cells).

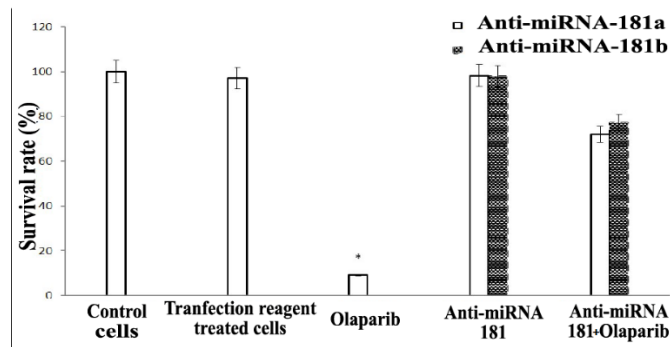

Figure (2): Survival rate of the transfected and un-transfected MBA-MB-231 cells with anti-miRNA-181a/b. * significantly decrease in survival rate at $P < 0.05$.
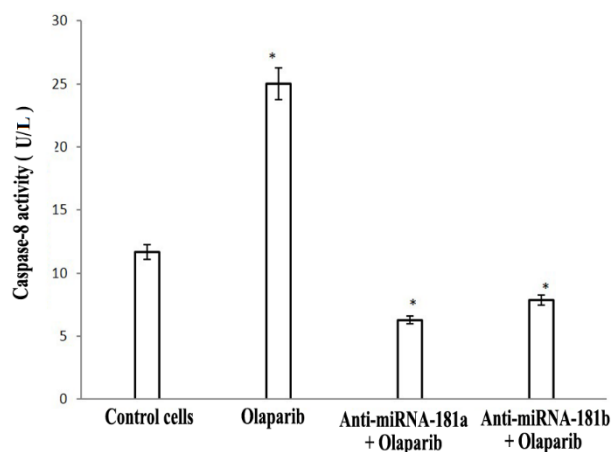

Figure (3): Level of caspase-8 activity in MDA-MB-231 cells transfected with either anti-miRNA-181a or anti-miRNA-181b and treated with (37.4 μg/ml) olaparib. * Significantly different compared to control (untreated cells) $P < 0.05$.
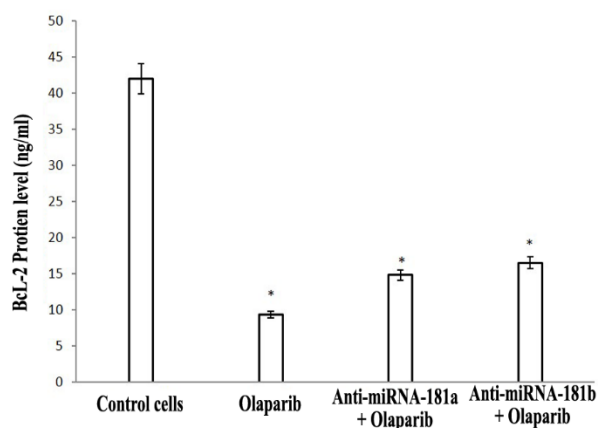

Figure (4): Level of BcL-2 protein in transfected and un-transfected MDA-MB-231 cells treated with (37.4 μg/ml) olaparib. * Significantly different compared to control (untreated cells) $P < 0.05$.
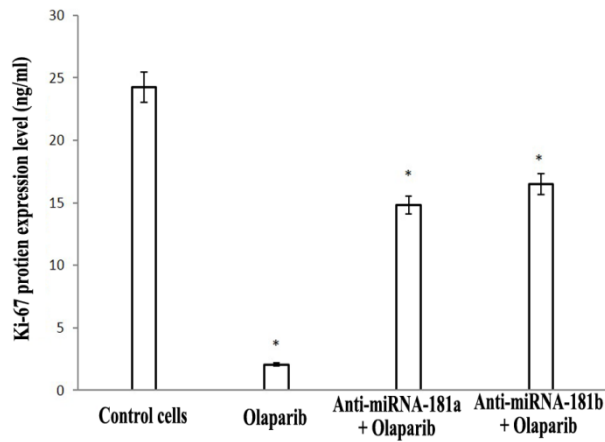
Figure (5): Effect of 48h treatment with (37.4 μg/ml) of olaparib on Ki-67 protein level in transfected and un-transfected MDA-MB-231 cells. * Significantly different at compared to control (untreated cells) $P < 0.05$.
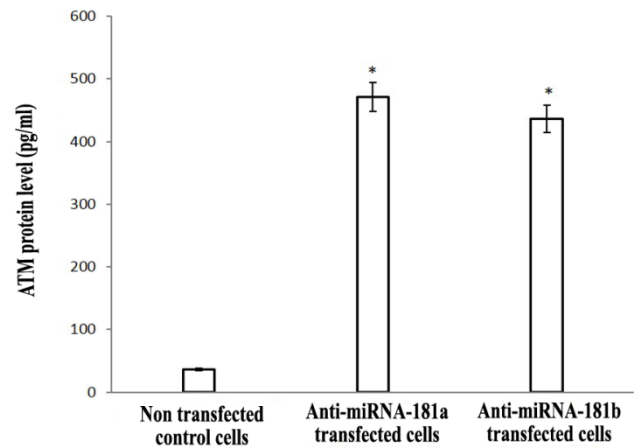

Figure (6): Effect of miRNAs-181a/b on ATM activity in transfected and un-transfected MDA-MB-231 cells. * Significantly different compared to control (untreated cells) $P < 0.05$.