Short thesis for the degree of doctor of philosophy (PhD)

Study the significance of miRNAs as biomarkers in the liquid biopsy of ovarian cancer

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The PhD defense will be organized online at 1:00 pm 27th of April, 2022. Participation requires registration. For registration and further information please email to marton.eva@med.unideb.hu until the 26th of April 2022, 4 pm.

1. Introduction

Ovarian cancer is the most lethal form of gynaecological malignancy that leads to more than 200.000 deaths annually (Sung et al., 2021). The highest incidence rates are observed in the developed parts of the world. This phenomenon might be explained by the fact that the lowest number of pregnancies represents an important risk factor for the development of ovarian cancer. Other risk factors include the presence of genetic mutations (e.g. *BRCA1, BRCA2*) or the long-term use of estrogen based hormone replacement therapy (Stewart et al., 2019). It is also important to highlight that the exposure to xenoestrogens [Zearalenone (ZEA) -mycotoxin; Bisphenol A (BPA) – synthetic compound used in plastic industry] might also increase the risk for gynaecological cancers (Rogowska et al., 2019; Sang et al., 2021).

The high mortality rate of ovarian cancer might be explained by the limitation of early detection. Currently, histopathological examination is considered to be the gold standard in the diagnosis of ovarian cancer. However, it is expensive, difficult to perform and it is not applicable for monitoring disease progression. Liquid biopsy is based on the detection of cell-free biomarkers present in body fluids that is considered to be a promising alternative in the non-invasive diagnostics of ovarian cancer. The advantages of this diagnostic method over tissue biopsy are: easier sampling, quicker diagnosis, and it is suitable for the follow-up of treatments and/or disease progression due to its repeatability. Furthermore, the quantity of cell-free biomarkers is not

affected by the heterogeneity of tumors that is a main limitation of tissue biopsies (Otandault et al., 2019).

Cell-free biomarkers might be cell-free nucleic acids including cell-free nuclear or mitochondrial DNA fragments as well as non-coding RNA molecules (e.g., miRNA) (Szilagyi et al., 2020). MicroRNAs are small (~19-25nt long), non-coding RNA molecules, which play important role in post-transcriptional gene regulation. MiRNAs have become the focus of interest in cancer studies recently due to the fact that they are involved in the regulation of cell proliferation, invasion and apoptosis. MiRNAs are considered to be promising biomarker candidates in cancer diagnostics due to the fact that miRNA expression highly differs in cancerous and healthy cells (Wang et al., 2018; Szilágyi et al., 2020). Screening miRNAs has several advantages: they are stable, they might be highly specific for different cancer types and their expression can be determined by various molecular biological methods e.g. by qPCR.

2. Aims

The application of CA125 and HE4 biomarkers in the diagnostics of ovarian cancer has not significantly improved the survival chances (Montagnana et al., 2017). Our aim was to identify cell-free miRNAs that might be promising biomarker candidates for ovarian cancer diagnostics. For this purpose, we performed the following experiments:

- Identification of biomarker candidates: we compared the cell-free miRNA expression (miR-200s, miR-34s, miR-203a and

miR-205) in the blood samples of patients suffered from ovarian cancer with healthy individuals by qPCR.

- Study the significance of miRNAs in ovarian cancer: we studied the biological relevance of miR-200s, miR-34s, miR-203a and miR-205 in estrogen-sensitive and non-sensitive human epithelial ovarian cell cultures.

3. Materials and Methods

Blood plasma samples

The total of 88 blood samples (60 from disease-free healthy controls and 28 from patients suffered from ovarian cancer) were used in the study. The samples were collected in EDTA tubes at the Department of Obstetrics and Gynecology. The study protocol was approved by the Scientific and Research Ethics Committee of the Medical Research Council (ETT TUKEB) [30231-2/2016/EKU]. Plasma was separated by two-step centrifugation and stored at -80 °C until further processing.

Cell lines and culturing conditions

We applied two human epithelial ovarian cell lines in our study. These were the estrogen sensitive PEO1 (ERα; ERβ), and the estrogen non-sensitive A2780 (ERβ) cell lines. Both of the cell lines were routinely cultured in RPMI1640 medium with 10% (PEO1) or 5% (A2780) FBS; 1% L-glutamine, 100 µg/mL streptomycin and 100 U/mL penicillin (37°C, 5% CO2). *Mycoplasma* contamination was tested by the PCR Mycoplasma Test Kit I/C (Promokine) before the experiments. After cell attachment, the medium was replaced by

PRF-RPMI1640 with 5% DCC-FBS and 1% L-glutamine. Cells were incubated for 24h, than E2, ZEA and BPA were added to the cultures in 1-1000 nM doses (37°C, 5% CO_2). In these experiments non-treated cells were applied as controls.

Phenotypic studies

In order to understand the effect of E2, ZEA and BPA molecules on cell proliferation cell count was determined after the treatments using a Bürker chamber. Their effect on migration was determined by wound-healing Scratch asssay. The toxic effect of these molecules was determined by the detection of apoptosis (study mitochondrial membrane potential by DilC1(5) assay) and cell lysis (determination of LDH activity in the supernatant of cell cultures by the CyQUANT LDH Cytotoxicity Assay Kit; Thermo Fisher Scientific).

Co-culture assay

In the co-culture experiment PEO1 cells were plated to Millicell cell culture inserts (0.4 μm pore size, Merck Millipore), that were placed to 24-well plates where A2780 cells had been plated previously. Transcriptional studies were performed 24 h and 48 h after co-culturing.

mRNA isolation and qPCR

RNA isolation was performed by RNeasy Mini kit (Qiagen). The expression of *ESR1* (estrogen receptor), *GREB1* (growth regulator), *CA12* (anhydrase), *DEPTOR* (mTORC target

protein), *RBBP8* (RB binding protein 8), *CDH1* (E-cadherin), *ZEB1* [a transcription factor involved in epithelial-mesenchymal transition (EMT)] genes was detected by QuantiTect SYBR Green RT-PCR Kit with the Lightcycler 96 PCR instrument. In these experiments *GAPDH* was used as a reference. Relative expression was determined by the ΔCt method.

Intracellular and cell-free miRNA isolation and qPCR

Total RNA including small RNAs was isolated from plasma samples and from the supernatant of cell cultures by miRNeasy serum/plasma kit (Qiagen). Intracellular miRNAs were extracted from cell lysates by miRNeasy Kit (Qiagen). Expression of miR-200a-3p, miR-200b-3p, miR-200c-3p, miR-141-3p, miR-429-3p, miR-34a-5p, miR-34b-3p, miR-34c-3p, miR-203a-3p and miR-205-5p was determined by the miScript workflow (miScript Primer Assays; miScript II RT Kit, miScript SYBR green PCR kit; Qiagen) with the Lightcycler 96 PCR instrument. In these experiments miR-103-3p was used as a reference. Relative expression level was determined by the ΔCt method.

Bioinformatics analysis of miRNAs

In order to understand the functional relationship between the studied miRNAs, target analysis [miRTargetLink (https://ccb-web.cs.uni-saarland.de/mirtargetlink/multinet.php; Hamberg et al., 2016)] and functional annotation of the target genes [DAVID tools (https://david.ncifcrf.gov/summary.jsp; Huang et al., 2009)] was performed.

Statistical analysis

Statistical tests were performed by the GraphPad Prism 8 and SPSS 25 statistical packages. The significance of miRNA expression in plasma samples was determined by Mann – Whitney U test. The correlation between miRNA expression in plasma samples was characterized by Spearmen correlation. The agreement of miRNA expression with CA125 and HE4 markers was studied by Cohen kappa test. The diagnostic potential of miRNAs was characterized by determining ROC-AUC, sensitivity, specificity, positive and negative predictive values as well as diagnostic accuracy. The results of cell cultures were analyzed by One-way ANOVA (post-hoc analysis: Dunnet test). Pairwise comparison of expression data was performed by Student's t-test using the ΔCt values.

4. Results

Identification of biomarker candidates

In the beginning of our work, we compared the expression of selected miRNAs (miR-200s, miR-34s, miR-203a and miR-205) in the plasma samples of patients suffer from ovarian cancer with the samples of healthy volunteers. According to these results, the relative expression of miR-200a, miR-200b, miR-200c, miR-141, miR-429, miR-34b, miR-203a ($p<0.001$), miR-205 ($p<0,01$) and miR-34a ($p<0.05$) proved to be significantly higher in the malignant samples than in healthy controls. However, we could not detect significant difference in the case of miR-34c between the two groups

(p=0.222). Diagnostic parameters were determined in the case of the differently expressed miRNAs. ROC-AUC was the lowest in the case of miR-34a (ROC-AUC: 0.655) and miR-205 (ROC-AUC: 0.683). This value proved to be the highest in the case of miR-200c (0.861). MiR-200a, miR-141, and miR-429 had the highest sensitivity (85.71%). Specificity proved to be the highest in case of miR-200b (90%), and the diagnostic accuracy was the highest in the case of miR-200b (82.95%). The agreement between the diagnostic tests based on the miRNAs and the standard biomarkers (CA125 and HE4) was also studied by Cohen kappa test. According to these results the agreement proved to be the highest between miR-200b and miR-200c (κ=0.889). It is also important to mention, that these miRNAs showed moderate agreement with HE4 (κ=0.400 and κ=0.481). However, the agreement of the studied miRNAs with CA125 was weak. According to our results, miR-200s and miR-203a might be promising biomarker candidates in the diagnosis of ovarian cancer, which is in good agreement with previous studies (Chen et al., 2019; Maeda et al., 2020). These miRNAs might be applied in multivariate diagnostic tests as well - e.g. by combining miR-200b and miR-200c with HE4 (Montagnana et al., 2017).

The functional relationship between these miRNAs was also studied by the correlation of their expression (Spearman correlation) and the number of their shared target genes. Those miRNAs, which expression showed strong correlation shared several targets. The correlation proved to be the highest between the expression of miR-200b and miR-200c (rs=0.774), which miRNAs shared 43 target

genes. Functional annotation of the target genes revealed that miR-200s, miR-34s, miR-203a and miR-205 co-regulate several genes involved in cell proliferation and migration. Furthermore, the target genes of miR-200s and miR-203a are also involved in the regulation of estrogen response.

Study the significance of miRNAs in ovarian cancer

In the second part of our work, the significance of miR-200s, miR-34s, miR-205 and miR-203a was studied in ovarian cells. For this purpose, we applied two human epithelial ovarian cell lines: the PEO1 (estrogen sensitive) and A2780 (estrogen non-sensitive). MiR-200s and miR-203a were strongly expressed in the cell lysates of the PEO1 cell line, among which miR-200b and miR-200c had the highest basal expression. However, the basal expression of miR-200s proved to be low in the ERα non-expressing A2780 cell line. The expression of miR-34a and miR-34b was hardly detectable in both cell lines. Similar phenomenon was observed when the cell-free expression of these miRNAs was studied. These results suggest that miR-200s, miR-203a, and miR-205 might have greater biological relevance in estrogen sensitive cells.

In order to strengthen our hypothesis, we examined the phenotypic response of PEO1 and A2780 cells to E2 and ZEA and BPA treatments. According to our results, E2, ZEA (1–100 nM) and BPA (10-100 nM) significantly increased the rate of cell proliferation in the PEO1 cell line that effect was not observable in the A2780 cell line. In the case of the cell migration E2, ZEA (10–

100 nM), and BPA (10 nM) increased the rate of migration in the PEO1 cell line. It is important to note that the presence of cell lysis or apoptosis was ruled out during the tested conditions. In agreement with our results E2, ZEA, and BPA increased cell proliferation and migration in several cell lines, which proved to be ERα dependent (Ptak et al., 2014; Andersen et al., 2017; Kowalska et al., 2018). It is also important to mention that the effect of ZEA was more comparable with E2 than the effect of BPA. Furthermore, ZEA and BPA induced phenotypic response at their physiologically relevant doses (1-10nM).

The effect of E2, ZEA, and BPA on gene expression was also studied. E2, ZEA, and BPA induced several estrogen-responsive genes including *GREB1* (Fc=263,19), *CA12* (Fc=37,01), *DEPTOR* (Fc=5,39), and *RBBP8* (Fc=9,58) in the PEO1 cell line that was not observable in the case of A2780. These studies also suggest that the effect of E2 was more comparable with ZEA, than with BPA. The expression of *CDH1* and *ZEB1* genes was also studied, which play key role in tumor cell invasion by mediating EMT. The *CDH1* gene was repressed (Fc=0,42) in response to estrogen treatments in the PEO1 cell line, that shows good agreement with the increased migratory ability of this cell line in response to estrogen exposure.

E2, ZEA and BPA treatments also affected miRNA expression in a time dependent manner. 12h after E2 exposure miR-200a (Fc=1,52), miR-200b (Fc=2,9), miR-200c (Fc=3,28), miR-141

(Fc=3,32) and miR-203a (Fc=1,73) were significantly overexpressed. However, the expression of miR-200b (Fc=0,28) and miR-200c (Fc=0,43) was downregulated 24h after estrogen exposure. The addition of ZEA and BPA also altered miRNA expression, although these changes were smaller. These results suggest that miR-200s and miR-203a might be important in the regulation of estrogen response that was also suggested by others (Klinge, 2015; Gao et al., 2019). This is highly remarkable in the respect of migration that is mediated by miR-200s through the inhibition of *ZEB1* that is involved in the transcriptional downregulation of *CDH1* expression (Koutsaki et al., 2014). As a consequence the down-regulation of miR-200s 24h after estrogen exposure might contribute to the repression of *CDH1* and increased migration of the PEO1 cell line. Similar phenomenon was observed previously in other cell lines (Park et al., 2008). Furthermore, higher basal expression of miR-200s and miR-203a in the PEO1 cell line and their early induction in response E2 treatment suggest the role of ERα in their transcriptional regulation. This hypothesis was confirmed by their decreased expression in response to MPP treatment – that is an ERα-selective antagonist.

Our results also suggest that cell-free miRNA expression correlated well with intracellular miRNA expression. According to the co-culture experiment the extracellular forms of miR-200b and miR-200c are able to influence the tumour microenvironment trough their transport into neighbouring cells. Similar phenomenon was also

demonstrated by others (Kogure et al., 2019). We suggest that monitoring cell-free miRNA expression in cell cultures might be applicable in monitoring intracellular miRNA expression and/or cellular physiology. Furthermore, it might be applied as an initial step in the search for biomarker candidates that might be further tested in body fluids.

5. Summary

Ovarian cancer is the most lethal form of gynaecological malignancy. The low survival rate is mainly due to the late diagnosis of the disease. Identification of non-invasive biomarkers might improve ovarian cancer diagnostics that would also contribute to the improvement of survival chances. MiRNAs are considered to be promising candidates for this purpose that are small, non-coding RNA molecules and might be involved in the development of cancer by the post-transcriptional regulation of genes involved in cell proliferation, migration or cell death. Furthermore, miRNAs are also detectable in body fluids that makes them promising candidates for liquid biopsy. Our aim was to identify miRNAs that might have high biological relevance in the development of ovarian cancer and could be good biomarkers in the diagnostics of this disease.

In the beginning of our work we identified 9 miRNAs, which were overrepresented in the plasma samples of patients suffered from ovarian cancer. Among these, 7 (miR-200a, miR-200b, miR-200c, miR-141, miR-429, miR-203a, miR-34b) had good diagnostic parameters. The expression of miR-200b and miR-200c

correlated well and the diagnostic tests based on these miRNAs showed high agreement with each other and medium agreement with HE4. We suggest that these miRNAs might be good candidates for the development of multivariable diagnostic tests in the future. We also studied the miRNA expression of estrogen sensitive and non-sensitive ovarian cell cultures. Our results showed that the miRNA expression of ovarian cells highly depends on the presence of ERα. MiR-200b and miR-200c might have high biological relevance in estrogen sensitive ovarian cells and their cell-free counterparts might have a high influence on the miRNA expression of surrounding cells. We conclude that these miRNAs might be good biomarkers for estrogen sensitivity of ovarian tumors that might increase the success of ovarian cancer therapy (e.g.: by the the application of estrogen disruptors). According to our phenotypic studies ZEA and BPA are able to increase cell proliferation and migration in ovarian cells that is highly comparable with the effect of E2. These results suggest the urgent need for more studies in this field in order to understand the carcinogenic effect of these molecules. We also suggest that monitoring cell-free miRNA expression in cell cultures could be applied as an initial step in the search for biomarkers which can be further tested in body fluids.

6. Main findings

• MiR-200a, miR-200b, miR-200c, miR-141, miR-429, miR-34a, miR-34b, miR-203a, and miR-205 were overrepresented in the plasma samples of patients suffered from ovarian cancer.

• MiR-200a, miR-200b, miR-200c, miR-141, miR-429, miR-34b and miR-203a had promising diagnostic parameters.

• The expression of miR-200b and miR-200c correlated well with each other and with HEA. These might be good candidates for the development of multivariable diagnostic tests in the liquid biopsy of ovarian cancer.

• The expression of miR-200a, miR-200b, miR-200c, miR-141, miR-429, miR-203a, and miR-205 was highly depended on the presence of ERα according to our studies with cell cultures.

• MiR-200b and miR-200c showed high basal expression in the ERα expressing PEO1 cell line, that suggests their high biological relevance in estrogen sensitive cells.

• The expression of miR-200a, miR-200b, miR-200c, miR-141 and miR-203a showed time-dependent induction to estrogen treatment in the PEO1 cell line. This effect was not observed when estrogen was applied with the ERα antagonist MPP that supports the role of ERα in their transcription.

• MiR-200b and miR-200c had high basal expression in PEO1 and were able to influence the miRNA expression of adjacent cells according to the results of co-cultures.

• According to our phenotypic and gene expression studies E2, ZEA and BPA were able to increase cell proliferation and migration in ERα expressing ovarian cells in physiologically relevant doses.

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9. List of publications

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List of publications related to the dissertation

1. **Márton, É.**, Varga, A., Soltész, B., Penyige, A., Lukács, J., Póka, R., Nagy, B., Szilágyi, M.: Comparative Analysis of Cell-Free miR-205-5p, let-7f-5p, and miR-483-5p Expression in Ovarian Cell Cultures and Plasma Samples of Patients with Ovarian Cancer.
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4. Szilágyi, M., Pös, O., **Márton, É.**, Buglyó, G., Soltész, B., Keserű, J., Penyige, A., Szemes, T., Nagy, B.: Circulating cell-free nucleic acids: main characteristics and clinical application.
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Total IF of journals (all publications): 28,108
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