Mutually distinguishing microRNA signatures of breast, ovarian and endometrial cancers *in vitro*

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Abstract. Early diagnosis and therapy in the first stages of a malignant disease is the most crucial factor for successful cancer treatment and recovery. Currently, there is a high demand for novel diagnostic tools that indicate neoplasms in the first or pre-malignant stages. MicroRNAs (miRNA or miR) are small non-coding RNAs that may act as oncogenes and downregulate tumor-suppressor genes. The detection and mutual discrimination of the three common female malignant neoplasia types breast (BC), ovarian (OC) and endometrial cancer (EC) could be enabled by identification of tumor entity-specific miRNA expression differences. In the present study, the relative expression levels of 25 BC, EC and OC-related miRNAs were assessed by reverse transcription-quantitative PCR and determined using the $2^{-\Delta\Delta Cq}$ method for normalization against the mean of four housekeeping genes. Expression levels of all miRNAs were analyzed by regression against cell line as a factor. An expression level-based discrimination between BC and OC cell types was obtained for a subgroup of ten different miRNA types. miR-30 family genes, as well as three other miRNAs, were found to be uniformly upregulated in OC cells compared with BC cells. BC and EC cells could be distinguished by the expression profiles of six specific miRNAs. In addition, four miRNAs were differentially expressed between EC and OC cells. In conclusion, miRNAs were identified as a potential novel tool to detect and mutually discriminate between BC, OC and EC. Based on a subset of 25 clinically relevant human miRNA types, the present study could significantly discriminate between these three female cancer types by means of their expression levels. For further verification and validation of miRNA-based biomarker expression signatures that enable valuable tumor detection and characterization in routine screening or potential therapy monitoring, additional and extended *in vitro* analyses, followed by translational studies utilizing patients' tissue and liquid biopsy materials, are required.

Introduction

The success rate in the clinical treatment of neoplastic disease remains highly associated with early detection of pre-malignant or first stages of malignant tissues. To date, only few highly specific and sensitive biomarkers are routinely used in the clinic for early-stage cancer screening or diagnostics.

Due to mammography screening, which was first introduced in 2005 in Germany, breast cancer (BC) has been identified at earlier stages, when treatment options are most promising and prognosis is most favorable (1). For endometrial cancer (EC) and ovarian cancer (OC), no standardized screening has yet been established. Postmenopausal bleeding serves as an early indicator of EC (2,3) European studies have shown that the 5-year survival rate of endometrial adenocarcinoma is >90% when detected at stage I compared with a survival rate of ~50% for advanced stages (II, III, IV) (3,4). OC remains one of the most challenging types of cancer to detect and treat. In most cases, tumor progression and metastasis are unnoticed until the advanced stages (5). According to the Surveillance, Epidemiology and End Results Program (National Cancer Institute, USA) database, the 5-year survival rate for localized disease is >90% in the USA population (6),

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Abbreviations: BC, breast cancer; EC, endometrial cancer; miRNA/miR, microRNA; OC, ovarian cancer

Key words: microRNA, breast cancer, endometrial cancer, ovarian cancer, diagnosis

however only 20% of ovarian cancer cases are detected at such an early stage in the USA (5,7).

One possible approach in the identification of novel potential biomarker candidates is based on expression profiling of different states, for example comparing malignant and healthy control expression profiles (7). In a stepwise filtering process, the discovery, qualification, verification, potential candidate prioritization and subsequent validation in adequate cohort sizes demonstrate the applicability of a biomarker for clinical practice implementation (7). Among a multitude of potential biomarker types, in previous years one group of nucleic acids has gained significant attention due to their diverse regulatory functions (8).

MicroRNAs (miRNAs or miRs) are small non-coding RNA molecules of ~22 nucleotides in length, which are involved in the post-transcriptional regulation of gene expression, predominately via gene silencing. By binding to various mRNA targets, upregulation of miRNA leads to reduced translation of mRNA or degradation of its transcript (9). In cancer, dysregulated miRNA expression plays an important role by upregulating oncogenes and downregulating tumor-suppressor genes, thus modulating cell proliferation, differentiation, apoptosis and stress response (10). The regulatory influence of miRNAs in breast and gynecological cancer biology has been demonstrated in a growing number of studies (8,11-17). The selection of miRNAs in the present study was based on an extensive literature search, with the major criterion being expression changes in the tumor types of BC, EC and OC (Table I), in combination with a proven detectability of all analyzed miRNA types in *in vitro* models as well as in human urine samples (18-20).

miRNA-21 (miR-21) is one of the most common miRNAs in epithelial cancer, and it generally promotes anti-apoptotic effects in various malignant tissues and cell lines, including BC, OC and EC, by downregulating tumor suppressors, such as phosphatase and tensin homolog (21,22) and programmed cell death protein 4 (23). In patients with BC, overexpression of miR-21 in the tumor is associated with advanced tumor stage, lymph node metastasis and poor survival (24). Whereas, in OC cell lines, miR-21 promotes pathways that enhance chemoresistance (25).

In contrast to miR-21, members of the miRNA family let-7 have most commonly been reported as tumor suppressors by downregulating Harvey rat sarcoma viral oncogene homolog and high-mobility group AT-hook 2 (26). However, studies have reported inconsistent results regarding the individual member let-7b. While some studies reported that high levels of let-7b in serum and plasma was associated with a favorable prognosis in cancer (27,28), a previous meta-analysis demonstrated reduced survival rates in high-grade serous OC with high tissue expression of let-7b (29). The tumor suppressing miRNA family miR-30 has been reported to exhibit pro-apoptotic effects by silencing ubiquitin-conjugating enzyme 9 and integrin β3 (30). In BC, miR-30a inhibits cell migration and invasion (31), whereas expression of miR-30c in tissues is associated with benefits during endocrine treatment (32) and regulatory effects in chemotherapy resistance processes (33). Notably, high expression levels of miR-30c and miR-30e have been observed in OC compared with normal tissue; however, both miRNAs are associated with an improved prognosis (34-36).

A more homogenous profiling has been observed for miR-125b and miR-100. miR-125b and miR-100 mediate the Erb-B2 receptor tyrosine kinase 2 and mechanistic target of rapamycin pathways, respectively, and downregulation of both miRNAs has been reported in BC, OC and EC tissue and cell lines (37-41). The previously described functional implications of the investigated miRNAs in BC, EC and OC tumor biology are summarized in Table I.

Due to recent investigations on miRNAs that are commonly conducted based on different study designs and environments, the comparison and interpretation of results between multiple cancer types have become increasingly challenging. The goal of the present study was to evaluate differences of miRNA profiling in three of the most common female cancer types: BC, OC and EC. Instead of solely focusing on individual miRNA types or families, the present study aimed to investigate the expression patterns of miRNAs that have great potential to serve as promising diagnostic tools in the distinction of different tumor types. Based on three cell types for each type of malignancy, BC, OC and EC, the detected differences in quantitative expression levels of a set of 25 miRNAs revealed diagnostic biomarker features clustered in tumor-entity-specific 'miRNA signatures'. To this end, the *in vitro* models used were selected to represent a range of common subtypes/properties of the respective carcinomas. The data obtained in this first phase biomarker identification study serve as a basis to prioritize distinct miRNAs with diagnostic significance that will be investigated in future studies.

Materials and methods

Cell culture conditions and treatments. The BC cell lines BT-20 (cat. no. 300130; CLS Cell Lines Service GmbH), BT-474 (cat. no. 00131; CLS Cell Lines Service GmbH) and SK-BR-3 (cat. no. 300333; CLS Cell Lines Service GmbH), the EC cell lines Ishikawa (cat. no. 99040201; Sigma-Aldrich; Merck KGaA), EFE-184 (cat. no. ACC 230; Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures GmbH) and AN3CA (cat. no. 300119; CLS Cell Lines Service GmbH), and the OC cell lines SK-OV-3 (cat. no. 300342; CLS Cell Lines Service GmbH), EFO-27 (cat. no. ACC 191; Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures GmbH) and OAW-42 (cat. no. 300304; CLS Cell Lines Service GmbH) were incubated in a humidified atmosphere at 37˚C and 5% CO_2. Ishikawa cells were cultured in RPMI-1640 (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% newborn calf serum (Gibco; Thermo Fisher Scientific, Inc.), 1% HEPES buffer (Gibco; Thermo Fisher Scientific, Inc.) and 100 U/ml Penicillin/Streptomycin (Sigma-Aldrich; Merck KGaA). The BT-20, SK-BR-2, EFE-184, AN3CA, SK-OV-3 and EFO-28 cells were cultured in DMEM/F12 (cat. no. 31331-028; Thermo Fisher Scientific, Inc.) supplemented with 10% newborn calf serum, 1% HEPES buffer and 100 U/ml Penicillin/Streptomycin. The BT-47 and OAW-42 cells were cultured in DMEM/F12 supplemented with 2.5% insulin (Insuman rapid®; Sanofi S.A.).

miRNA isolation. miRNA from cultured cells was isolated using the innuPREP Micro RNA kit (Analytik Jena US LLC), according to the manufacturer's instructions. Isolated RNA

Table I. miRNA types with functional implications in breast, endometrial and ovarian cancers.

miRNA	Target genes	Breast cancer	Endometrial cancer	Ovarian cancer	(Refs.)
Let-7a	CCL21, CCR7, RAS, HMGA-11, HMGA-2, cyclin A2, CDC34, STK6, STK12, E2F5, CDK8, CDC25A, CDK6, Casp3, Bcl2, Map3k1, Cdk5	Plasma ↑ (53) Tissue ↓ (54,55) Cell line ↓ (54-56) Tissue ↓ (57)	Tissue ↓ (58)	Cell line ↓ (59)	(28,56-61)
Let-7b		Tissue ↓ (57)	Cell line ↓ (60)	Tissue ↓ (61) Serum ↓ (28) Cell line ↓ (59)	(28,60,62-64)
Let-7c			Tissue (endometrioid) ↑ (62) Tissue (Sarcoma, mixed epithelial-mesenchymal tumors) ↓ (63)	Cell line ↓ (59)	(28,65,66)
miR-21	ANP32A, BTG2, Bcl2, P12/CDK2AP1, HNRPK, IL-12p35, JAG1, MEF2C, hMSH2, PDCD4, PTEN, RECK, RhoB, SMARCA4, TGFBRII	Tissue ↑ ((53,64) CTCs ↑ (64) Plasma ↑ (64) Cell lines ↑ (65)	Cell line ↑ (22)	Cell line ↑ (66)	(19,56,67-69)
miR-27a	Wnt, PPARγ, C/EBPα, FOXO1	Tissue ↓ (65)	Tissue (67) Cell lines ↑ (67)	Cell lines ↑ (46)	(46,68,70)
miR-30a	ITGB3, UBC9, TP53, TRADD, CCNE1 (NF-κB)	Cell lines ↓ (65,68,69) Tissue → (70)	Cell line ↓ (60) Tissue ↓ (71) Plasma ↓ (71)	Cell line (Clear Cell) ↑ (72) Tissue (Clear cell) ↑ (73) Urine ↑ (74) Plasma ↓ (75)	(57,68,71,76)
miR-30c		Tissue ↓ (77)	Tissue ↓ (62,78)	Tissue ↑ (34) Whole blood ↑ (76) Cell line (drug-resistant) ↓ (79)	(31,65,79-82)
miR-30e		Plasma ↓ (53) Tissue ↓ (53)	Tissue (papillary serous vs. Endometrioid) ↑ (79)	Tissue ↑ (35)	(31,56,82)
miR-100	mTOR, PLK-1, FRAK1, Wnt/β-Catenin	Cell lines ↓ (80) Tissue ↓ (38)	Tissue ↓ (38)	Tissue ↓ (38) Serum ↓ (81)	(35,82,83)
miR-7	EGFR, IGF1R, PIK3CD, KLF4	CSCs ↓ (17,82)	Tissue ↑ (17)	Tissue ↑ (83)	(17,37,84)
miR-125b	ERBB2, ERBB3, BCL3, EPOR	Tissue ↓ (65)	Cell line ↓ (37)	Tissue ↓ (83) Serum ↓ (81)	(68,83,81,84)
miR-9	REST, CoREST	Cell lines ↑ (65)	Tissue ↑ (67) Cell lines ↑ (67) Plasma ↓ (15)	Tissue ↑ (83)	(15,59,75,84)
miR-15b	BCL2, CHEK1	Cell lines ↓ (65)	Tissue ↓ (84)	Tissue ↓ (83)	(68,84,85)
miR-128.1	R3HDM1, RCS	Cell lines ↓ (65)	Tissue ↑ (67) Cell lines ↑ (67)	Cell lines ↓ (81)	(68,75,86)

Table I. Continued.

miRNA	Target genes	Breast cancer	Endometrial cancer	Ovarian cancer	(Refs.)
miR-222	CD117, PBX3	Cell lines ↑ (65) Serum ↑ (65)	Serum ↑ (17)	Tissue ↓ (83)	(17,68,84)
miR-29	STAT3, MCL1, TCL1A, TTP, DNMT3	Cell lines ↑ Serum ↑ (65)	Tissue ↓ (84)	Serum ↑ (85)	(68,85,87)
miR-92a	ERβ, MUC16	Cell lines ↓ (65)	Tissue ↑ (15) Plasma ↑ (15)	Serum ↑ (85)	(15,68,87)
miR-200	ZEB1, ZEB2, β tubulin III	Cell lines ↓ (65) Tissue ↑ (57)	Cell lines ↑ (17,86) Tissue ↑ (15,84) Plasma ↑ (15)	Tissue ↑ (83) Serum ↑ (81)	(15,17,60, 68,83-85,88)
miR-17	NOR-1, GALNT3	Cell lines ↓ (87)	Cell lines ↓↑ (86)		(88,89)
miR-20	NOR-1, PTEN/PI3K/Akt	Cell lines ↓ (87)	Cell lines ↑ (86) Tissue ↓ (84)	Tissue ↓ (83)	(84,85,88,89)
miR-19b	Mfn1, PITX1, ATXN1, PTEN, Sbf2, Bcl7a, Rnf44	Cell lines ↓ (87)	Cell lines ↓ (86)	Tissue ↑ (88)	(88-90)
miR-106b	E2F5, SLC2A3, E2F1, TWST1	Cell lines ↓ (87)	Tissue ↑ (17)	Tissue/Cell line (89)	(17,89,91)
miR-221	CD117, SND1, AEG-1	Cell lines ↑ (65) Serum ↑ (65)	Tissue ↓ (17, 84)	Serum ↑ (85) Tissue/Cell line (89)	(17,68,85,87,91)

↑, upregulated expression; ↓, downregulated expression; miR/miRNA, microRNA.

was quantitatively determined using the NanoDrop ND1000 (VWR International GmbH). RNA samples were stored at -20˚C until further processing.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was isolated using GeneMATRIX Universal RNA/miRNA Purification kit (cat. no. E3599; EURx®; Roboklon GmbH) according to manufacturer's protocol. A total of 1 μg isolated RNA per sample was used for RT. The RT reaction mix contained 5 μl RT-buffer (5X), 1 μl 2.5 μM poly A adapter primer (Apara Bioscience GmbH), 0.5 μl 5 mM dNTPs (Jena Bioscience), 0.25 μl Maxima reverse transcriptase (Thermo Fisher Scientific, Inc.), 0.25 μl SUPERase In RNase inhibitor (Thermo Fisher Scientific, Inc.), 0.5 μl 10 mM ATP (New England Biolabs, Inc.), 0.25 μl poly A polymerase, and 1 μg RNA sample. The reaction was performed on a thermal cycler (Eppendorf) at 37˚C for 60 min and stopped at 85˚C for 10 min. Processed cDNA was stored at 4˚C.

The relative expression levels of specific miRNAs were assessed by qPCR using the SYBR-Green assay in a duplicate analysis. A total of 1 μl cDNA per sample with a concentration of 5 ng/μl was mixed with 9 μl Master Mix, containing 1 μl buffer (10X), 0.5 μl 5 mM dNTPs (Jena Bioscience GmbH), 0.5 μl 5 μM primer (Apara), 0.5 μl SYBR-Green (Roche Diagnostics), 0.05 μl HotStart Taq (Jena Bioscience GmbH) and 6.45 μl nuclease-free water (Analytik Jena US LLC). The primer pairs consisted of a universal reverse primer (30-32) and a specific miRNA sense primer. The qPCR was performed on a LightCycler® 480 instrument (Roche Diagnostics) at 95˚C for 5 min, followed by 40 cycles at 95˚C for 5 sec, 62˚C for 15 sec and 72˚C for 10 sec. Data were analyzed with the LightCycler® 480 software (Roche Molecular Systems, Inc.; Version 1.5.1). The relative expression of each miRNA was determined using the $2^{-\Delta\Delta Cq}$ method (42,43) based on the housekeeping genes small nucleolar RNA, C/D box 48 (RNU48), miR-26b, miR-16 and miR-103, with the 'BestKeeper' software tool (Version 1) (43). The specific primer sequences are listed in Table II.

Statistical analysis. The expression levels of all BC, EC and OC-associated miRNAs were determined as mean ΔC_q values of the miRNA normalized against the geometric mean of the four housekeeping genes RNU48, miR-16, miR-26b and miR-103. The expression levels of all miRNA types were separately analyzed using a linear model with cell line as the independent variable. The regression coefficients with 95% confidence intervals were tabulated. This led to color coded heatmaps in which red colors indicate strong deviations in the positive direction and blue colors indicate strong deviations in the negative direction from the expression level in the cell line that served as a reference (AN3CA, BT-474 and BT-20). Dark colors correspond to a $P<0.00005$, and light colors correspond to a $P<0.00025$. All other comparisons are presented in gray.

Table II. Primer sequences.

Primer	Sequence (5'→3')
miRNA poly A RT	GAAGACTCAGTTGCACTCTACCAAT TAAGACGAACAGAGCCATACTTTTT TTTTTTTNN
Universal antisense	GACTCAGTTGCACTCTACCAATTAA
miR-16-5p	GGCTAGCAGCACGTAAATATTG
miR-26b-5p	GGCGTTCAAGTAATTCAGGATAG
RNU48	TGTGTCGCTGATGCCATC
miR-103-5p	CGGAGCAGCATTGTACAGG
let-7a-5p	CGGTGAGGTAGTAGGTTGTATAGTT
let-7b-5p	CGTGAGGTAGTAGGTTGTGTG
let-7d-5p	CGGAGAGGTAGTAGGTTGCATA
miR-7-5p	CGGTGGAAGACTAGTGATTTTGTT
miR-9-5p	CGGTCTTTGGTTATCTAGCTGTAT
miR-15b-5p	GCTAGCAGCACATCATGGTTTA
miR-17-5p	GCAAAGTGCTTACAGTGCAG
miR-19b-3p	GTGTGCAAATCCATGCAAAACT
miR-20a-5p	CGGTAAAGTGCTTATAGTGCAGGTA
miR-20b-5p	CAAAGTGCTCATAGTGCAGGTA
miR-21-5p	GGCTAGCTTATCAGACTGATGTT
miR-27a-3p	GGCTTCACAGTGGCTAAGTT
miR-29a-3p	GTAGCACCATCTGAAATCGGTT
miR-30a-5p	GTGTAAACATCCTCGACTGGAA
miR-30c-5p	GCTGTAAACATCCTACACTCTCA
miR-30e-5p	GGTGTAAACATCCTTGACTGGAA
miR-92a-3p	GTATTGCACTTGTCCCGGC
miR-100-5p	GAACCCGTAGATCCGAACTT
miR-106b-5p	GCTAAAGTGCTGACAGTGCA
miR-125b-5p	GCTCCCTGAGACCCTAACTT
miR-128-1-3p	GTCACAGTGAACCGGTCTCTT
miR-200b-3p	CGGTAATACTGCCTGGTAATGAT
miR-200c-3p	CGTAATACTGCCGGGTAATGAT
miR-221-3p	GCTACATTGTCTGCTGGGTT
miR-222-3p	CGAGCTACATCTGGCTACT

miR, microRNA.

Results

miRNA expression profiles of the cell lines. In the present study, the expression levels of 25 BC, EC and OC-associated miRNAs (let-7a, let-7b, let-7d, miR-7, -9, -15b, -17, -19b, -20a, -20b, -21, -27a, -29a, -30a, -30c, -30e, -92a, -100, -106b, -125b, -128.1, -200b, -200c, -221, -222) were quantified in three BC, EC and OC cell lines. The characteristics of each cell line are presented in Table III.

The statistical analyses demonstrated that comparing the three different cell types (AN3CA, BT-474 and BT-20) revealed a range of moderately to highly differentially expressed miRNAs, which exhibited either marked upregulation or downregulation. By clustering miRNAs with respect to their differential expression characteristics, subgroups of miRNAs featuring potential biomarkers to discriminate between BC, OC and EC cells could be created. The expression data clearly revealed a BC-associated miRNA subpanel with significantly distinct expression levels compared with the gynecological tumor types EC and OC (miRs: let-7b, -21, -27a, -30a, -30c, -30e). Consecutively, miRNA clusters with

Table III. Cell line characteristics.

Cell line	BC subtype classification	Receptor status	Primary tumor	Origin	Other characteristics	(Refs.)
BT474	Luminal B	ER^+, $PR^{+/}$, $HER2^+$	Invasive ductal carcinoma	Breast	Ki-67 high, normally endocrine responsive, variable to chemo therapy response, trastuzumab responsive	(90-94)
SK-BR-3	HER2	ER^-, PR^-, $HER2^+$	Invasive ductal carcinoma	Metastasis (pleural effusion)	Ki-67 high, trastuzumab respon sive, chemotherapy responsive	(90,92-95)
BT-20	Triple-negative	ER^-, PR^-, $HER2^-$	Basal A-like invasive ductal carcinoma	Breast	Docetaxel responsive, tamoxifen responsive	(94-97)
Ishikawa	$LHRH^+$	ER^+, PR^+, AR^+	Endometrial adeno-carcinoma	Endometrium	Tamoxifen-responsive	(96-98)
EFE-184		ER^+, PR^-	Carcinoma (relapse)	Endometrium from ascites fluid	Tamoxifen-responsive	(56,99)
AN3CA		ER^-, PR^+	Undifferentiated endometrial adenocarcinoma	Lymph node metastasis	Non-steroid response	(98-101)
SK-OV-3	$LHRH^-$		Ovarian adenocarcinoma	Ascites fluid	Resistance of TNF, cisplatin-responsive, upregulated expression of FGF16 and PITX2	(100-104)
OAW-42			Adenocarcinoma	Ascites fluid	Upregulated expression of FGF16 and PITX2, tamoxifen responsive	(100,102-104)

ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor 2; Ki-67, marker of proliferation Ki-67; TNF, tumor necrosis factor; FGF16, fibroblast growth factor 16; PITX2, pituitary homeobox 2.

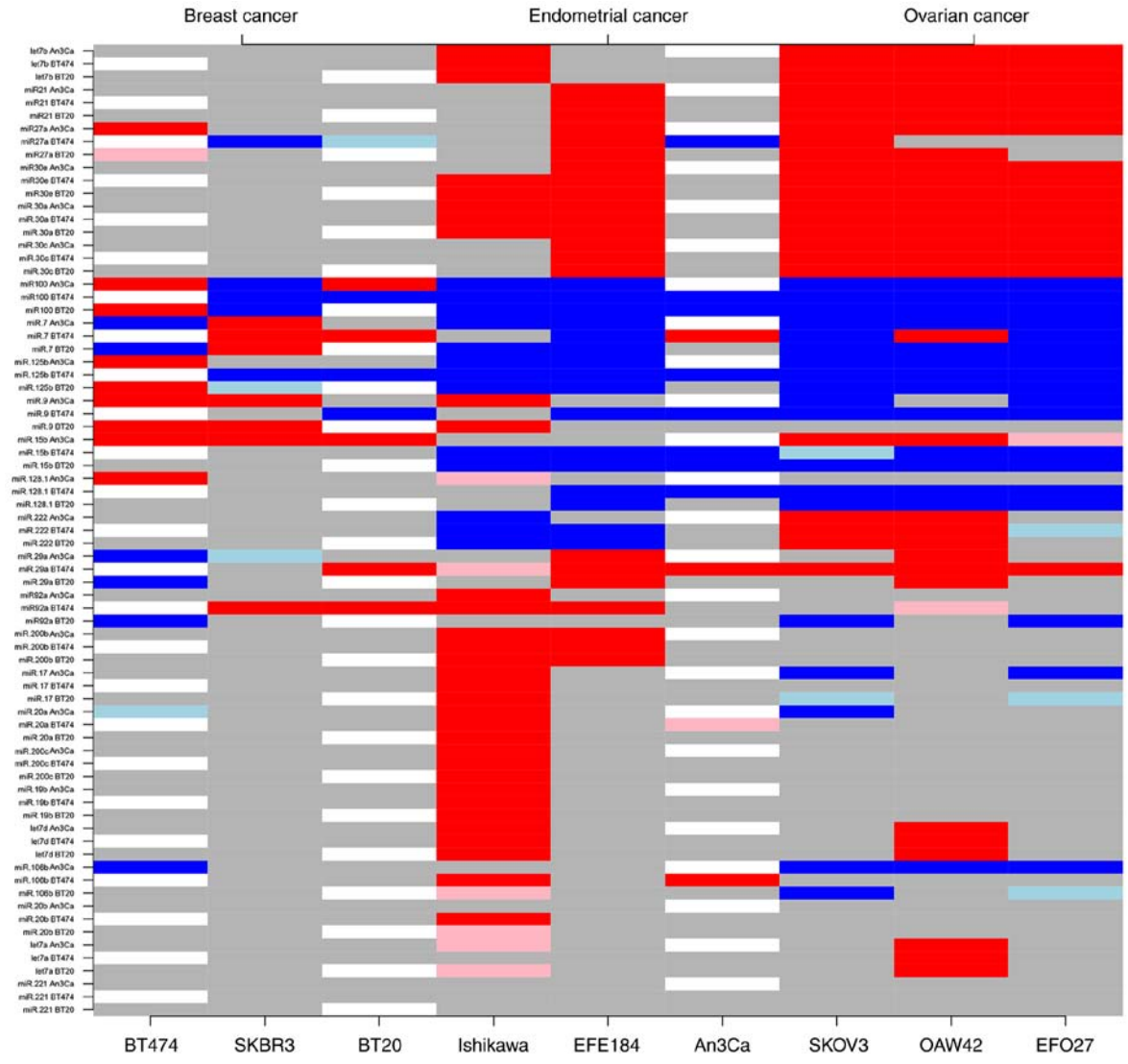

Figure 1. Mutual expression differences of distinct miRNA types in breast, endometrial and ovarian cancer cell lines. The colors indicate either miRNA overexpression on a significant (light red, P<0.00025) or highly significant (red, P<0.00005) level, or decreased miRNA expression levels on a significant (light blue, P<0.00025) or highly significant (blue, P<0.00005) level. Gray indicates insignificant expression differences, and white indicates the reference parameter. miRNA, microRNA.

statistical relevance were defined to allow for discrimination between the three tumor entities in a one-versus-one approach (Fig. 1 and Tables SI-SIII).

miRNAs discriminating BC from OC cells. Expression analyses could determine a subgroup of ten different miRNAs (miRs: let-7b, -21, 30a, -30c, -30e, -27a, -222, -29a, -128.1, -9) that facilitated an expression level-based discrimination between the BC and OC cell types. The notable types included let-7b, miR-21 and the miR-30 family genes, which were uniformly upregulated in OC cells compared with BC cells. For example, compared with AN3CA cells, miR-let-7b was upregulated by a mean value of 5.08 (95% confidence interval, 4.51, 5.65; P<0.001) in SK-OV-3, 8.37 (7.80, 8.94; P<0.001) in OAW-42 cells, and 2.53 (1.96, 3.10; P<0.001) in EFO-27 OC cells (Table SI). In contrast, regression analyses demonstrated no significant difference of miR-let-7b in all investigated BC cell lines (Fig. 2 and Table SII). The expression levels of miR-30a, miR-30c and miR-30e were also increased in all three investigated OC cell lines. Specifically, compared with AN3CA cells, the miR-30a was significantly increased by a mean value of 0.22 (0.21, 0.23; P<0.001) in SK-OV-3, 0.12 (0.10, 0.13; P<0.001) in OAW-42 cells, and 0.43 (0.42, 0.44; P<0.001) in EFO-27 OC cells. The expression levels of miR-30c and miR-30e were also upregulated by a mean value of 0.21 (0.17, 0.25; P<0.001) and 0.07 (0.06, 0.08; P<0.001) in SK-OV-3 cells, 0.12 (0.08, 0.16; P<0.001) and 0.03 (0.02, 0.04; P<0.001) in OAW-42 cells, and 0.50 (0.46, 0.55; P<0.001) and 0.16 (0.15, 0.17; P<0.001) in EFO-27 OC cells (Table SI). No significant differences were identified among all BC cell lines (Table SII). miR-27 and miR-29a exhibited a moderate downregulation in BC cells, with few inconsistent results depending on the cell line comparison (AN3CA or BT-474). By contrast, miR-9 and miR-128.1 exhibited a general moderate downregulation in OC cells compared with BC cells (Fig. 2 and Tables SI and SII).

miRNAs discriminating BC from EC cells. Among the 25 miRNAs evaluated in the present study, six exhibited

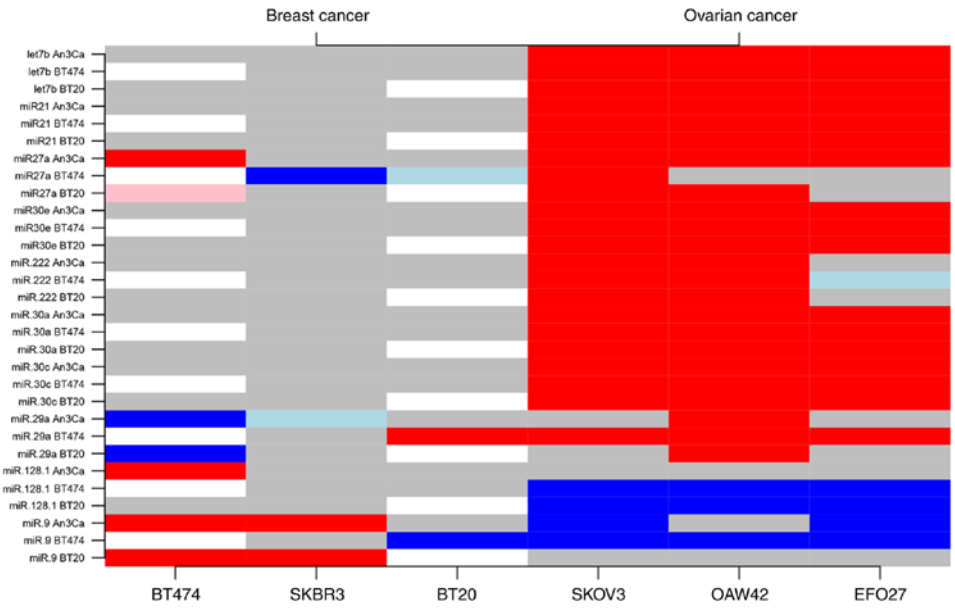

Figure 2. Mutual expression differences of miRNA types in the comparison of breast vs. ovarian cancer cell lines. The colors indicate either miRNA overexpression on a significant (light red, P<0.00025) or highly significant (red, P<0.00005) level, or decreased miRNA expression levels on a significant (light blue, P<0.00025) or highly significant (blue, P<0.00005) level. Gray indicates insignificant expression differences, and white indicates the reference parameter. miRNA, microRNA.

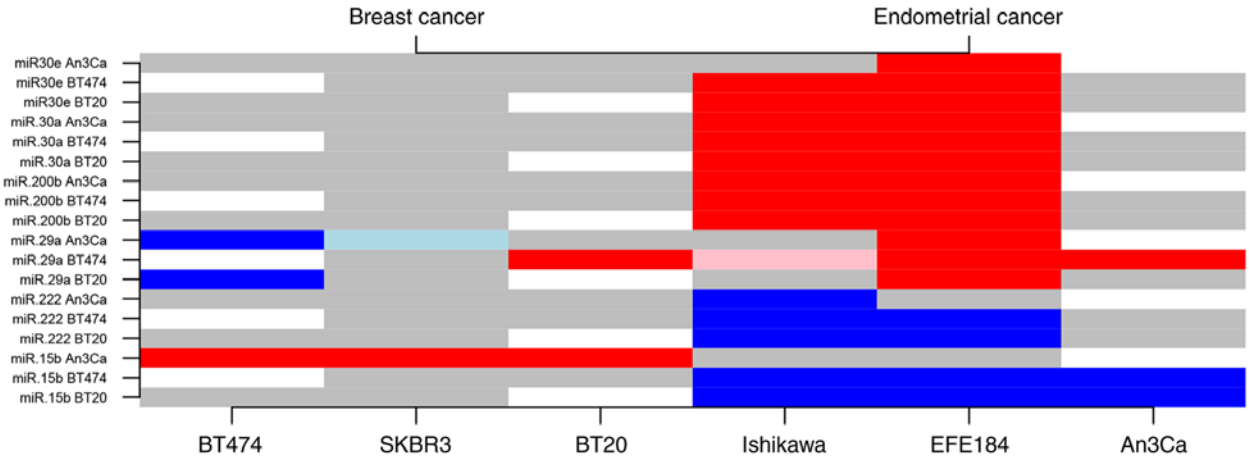

Figure 3. Mutual expression differences of miRNA types in the comparison of breast vs. endometrial cancer cell lines. The colors indicate either miRNA overexpression on a significant (light red, P<0.00025) or highly significant (red, P<0.00005) level, or decreased miRNA expression levels on a significant (light blue, P<0.00025) or highly significant (blue, P<0.00005) level. Gray indicates insignificant expression differences, and white indicates the reference parameter. miRNA, microRNA.

distinguishing characteristics in regard to BC compared with EC cell expression profiles (miRs: -30a, -30e, -29a, -15b, -200b, -222). While miR-29a, -30a, -30c and -200b were found to be upregulated in EC cells, miR-15b and miR-222 demonstrated downregulated expression levels in comparison to BC cells. For example, miR-200b was upregulated by a mean value of 5.43 (5.27, 5.58; P<0.001) in Ishikawa cells and by 0.97 (0.81, 1.12; P<0.001) in EFE-184 EM cells. Notably, AN3CA cells did not fully comply to the EC-specific expression level trends, which may be explained by cell-specific molecular characteristics (Fig. 3 and Tables SII and SIII).

miRNAs discriminating OC from EC cells. A total of four miRNAs (miR-92a, -106b, -200b, -222) with altered expression levels that may serve a role in the determination of endometrial compared with ovarian malignancies were identified based on this *in vitro* approach. Upregulated expression levels of miR-92a, -106b and -200b in EC cell types, as well as an upregulation of miR-222 in OC cells may help to mutually distinguish between these tumor types. Compared with AN3CA cells, miR-222 expression was increased by a mean value of 0.66 (0.52, 0.80; P<0.001) in SK-OV-3 cells and by 0.85 (0.71, 0.99; P<0.001) in OAW-42 OC cells. By contrast, a downregulation was identified in two EC cell lines by a mean value of 0.48 (-0.62, -0.34; P<0.001) in Ishikawa cells and by 0.18 (-0.32, -0.04; P=0.018) in EFE-184 EM cells. However, individual cell line-specific differences need to be taken into account in the assessment of tumor type determination of a

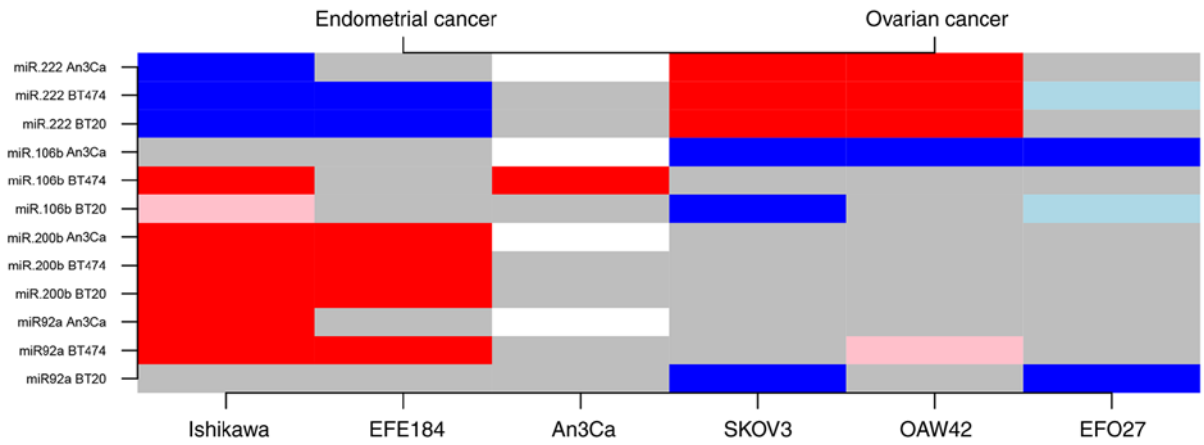

Figure 4. Mutual expression differences of miRNA types in the comparison of endometrial vs. ovarian cancer cell lines. The colors indicate either miRNA overexpression on a significant (light red, P<0.00025) or highly significant (red, P<0.00005) level, or decreased miRNA expression levels on a significant (light blue, P<0.00025) or highly significant (blue, P<0.00005) level. Gray indicates insignificant expression differences, and white indicates the reference parameter. miRNA, microRNA.

potential miRNA subpanel with diagnostic power in this regard (Fig. 4 and Tables SI and SIII).

Discussion

The search for clinically applicable biomarkers necessitates a stringent multi-step selection process to singularize, evaluate and validate the usability of a potential biomolecule, or even grouped biomolecule expression profiles or signatures for clear diagnostic purposes. The present study focused on one possible initial step in the determination of potential novel biomarkers that help to detect and distinguish healthy women from patients with malignant disease of the breast, endometrium or ovaries. Based on an *in vitro* model approach, the proof of principle was accomplished to corroborate the initial hypothesis of discriminating diagnostic features of miRNA signatures in the diagnosis of breast and gynecological malignancies. In general, the detected intracellular miRNA expression levels can be transferred to the extracellular setting of secreted miRNAs, as shown in a previous study (20). Therefore, the experimental design of the present study was targeted on the identification of miRNA signatures, based on tumor-specific expression differences, that enable the mutual discrimination of the three common female cancer types BC, OC and EC. Since miRNAs are robust and easily accessible biomolecules that can be quantified in a wide range of biomaterials, including tissue and liquid biopsies, they meet important requirements for modern and applicable diagnostic biomarkers (44). Thousands of different human miRNAs have been described, of which a clinically relevant subset of 25 different miRNAs with potential impacts in BC, EC and/or OC was pre-selected for the present analytical *in vitro* approach. Although certain differences in cell line-based and *in vivo* settings need to be kept in consideration, the current study is intended to provide initial findings that guide further investigations in a promising direction.

Global expression profile analyses in the present study resulted in the identification of cancer type-specific miRNA subgroups. These clusters of distinct miRNAs were characterized by differences in expression levels that can significantly discriminate between the tumor types BC, OC and EC. However, no significant subtype-specific miRNA expression signature differences could be detected among the respective cancer types analyzed.

A parallel comparison of entity-specific clustering habits highlighted a BC-specific miRNA subpanel of six miRNAs that exhibited significantly different expression levels compared with those observed in EC and OC *in vitro* models. In particularly, members of the miR-30 family were identified in this respect.

Comparisons of miRNA expression signatures in either BC/OC, BC/EC or EC/OC clearly revealed the most miRNA expression profile differences in the comparison of BC vs. OC, with ten of the 25 miRNAs exhibiting significantly different expression levels in these tumor types. Members of the miR-30 family were identified to be significantly differentially expressed, in addition to few more types, including miR-9, which has previously been described as a prognostic marker in OC (45), as well as miR-222 and miR-29a, which are known triggers in breast cancer therapy resistance mechanisms (46). In previous studies, the let-7 family has been reported to exhibit decreased expression levels in OC tissues as well as in OC cell lines, and has been identified to serve a role in OC progression (47,48). In contrast to the literature, in the present study, let-7b was found to be upregulated in OC cells compared with BC cells.

A direct comparison of EC and BC miRNA expression signatures revealed six miRNAs with significantly different expression levels. Again, members of the miR-30 family were prominent, but also miR-15b and mi-200b were identified in this comparison. miR-15b has been described as an aberrantly regulated tumor suppressor (49), whereas miR-200b has a role in epithelial-mesenchymal transition processes (50).

The miRNAs miR-92a, miR-106b, miR-200b and miR-222 compose the smaller subgroup of four miRNAs that exhibited significant expression differences in EC compared with OC *in vitro* models. Consistent with a previous study by Záveský *et al* (51), the present data confirmed the differential expression of the miRNAs miR-92a, miR-106b and miR-200b in EC compared with OC. Upregulated expression levels of

miR-222 in OC cells were found to associated with epithelial OC in a previous investigation (52).

In conclusion, the diagnostic power and validity of entity-specific miRNA clusters is partially limited due to individual cell type characteristics, such as receptor status or tumor origin (primary tumor or metastasis). For instance, in the present study the estrogen receptor (ER) EC cell line exhibited a different miRNA expression compared with two ER^+ EC cell lines. In addition, EFO-27 deviated from the other OC *in vitro* models to a certain extent, thus an intra-entity variation in miRNA expression signatures has to be taken into account. Furthermore, the present analyses revealed a notable difference in the molecular relationship of EC and OC compared with BC. Therefore, the number of miRNAs with distinguishing expression levels was markedly increased in EC and OC vs. BC than in the comparison between EC vs. OC.

To pursue the identification of a clinically valuable highly entity-specific signature panel with diagnostic power for implementation in routine screening, the obtained data of the present discovery phase approach require further verification and validation. Thus, additional and extended *in vitro* analyses, followed by translational studies using patients' tissues and liquid biopsy materials should be performed in further analyses to provide substantial evidence for miRNA-based biomarker expression signatures that enable tumor detection, characterization and potential therapy monitoring.

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Availability of data and material

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

The project idea and experimental design was conceived by MH, MJ, DW, SM, BK and TE. *In vitro* experiments were performed by MJ, CN and DW. Data and statistical analyses were performed by GR, supported by DW, TE and MH. MH, IG, JW, TE, GR, MV, KB and JA interpreted the results and wrote the manuscript. GR, MV, BK, KB and SM critically revised the final version of the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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