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Circulating miRNA Profiling of Women at High Risk for Ovarian Cancer

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Abstract

Survival of epithelial ovarian cancer patients remains poor without significant change over many decades. There is a need to better identify women at high risk (HR) for ovarian cancer. We propose that miRNA dysregulation may play critical roles in the early stages of ovarian cancer development. Circulating miRNAs may represent an important biomarker in this context, and miRNA profiling of serum in women at HR compared to those at low risk (LR) may give insights in tumor initiation pathways. There is also rationale for a specific focus on regulation of the androgen and its related hypoxia pathways in tumor initiation. We hypothesized that subsets of these pathway related miRNAs may be downregulated in the HR state. Serum from four HR and five LR women were sequenced and analyzed for 2083 miRNAs. We found 137 miRNAs dysregulated between the HR and LR groups, of which 36 miRNAs were overexpressed in HR and the vast majority (101 miRNAs or 74%) downregulated in the HR, when compared to LR serum. mRNA targets for the differentially expressed miRNAs were analyzed from three different miRNA-mRNA interaction resources. Functional association analysis of hypoxia and androgen pathway mRNA targets of dysregulated miRNAs in HR serum revealed that all but one of the miRNAs that target 52 hypoxia genes were downregulated in HR compared to LR serum. Androgen pathway analysis also had a similar expression pattern where all but one of the miRNAs that target these 135 identified genes were downregulated in HR serum. Overall, there were 91 differentially expressed miRNA-mRNA pairings in the hypoxia analysis. In the androgen-related analysis, overall, there were 429 differentially expressed miRNA-mRNA pairs. Our pilot study suggests that almost all miRNAs that are conserved and/or validated are downregulated in the HR compared to LR serum. This study, which requires validation, suggests that, via miRNA dysregulation, involvement of both hypoxia and its related androgen pathways may contribute to the HR state. This pilot study is the first report to our knowledge that studies circulating miRNA profiling of HR and LR women.

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Introduction

Women at high risk (HR) for epithelial ovarian cancer are at risk for development of high-grade serous ovarian, fallopian tube, and primary peritoneal carcinomas, collectively called "ovarian" cancer. These women include those carrying BRCA or other genetic mutations that predispose to differing levels of ovarian cancer risk but also include those who may be at risk solely based on personal and family history. BRCA mutations result in defective homologous recombination repair of DNA and thereby enhanced ovarian cancer risk. Those at HR may have between a 5% and 50% lifetime risk of

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developing ovarian cancer. Epithelial ovarian cancer survival remains poor without significant change over many decades. There is a need to more specifically identify the HR population that may allow for early detection or for optimal timing of risk-reducing surgery to prevent development of ovarian cancer in these women.

MicroRNAs (miRNAs) are frequently dysregulated in cancer and disease. By binding to mRNAs of critical proteins, miRNAs posttranscriptionally regulate protein expression, thereby influencing downstream pathways. Frequently, they result in RNA silencing of target gene expression. Circulating miRNA profiling studies have found miRNAs that are differentially expressed between ovarian cancer patients and those who are healthy controls [1–5] or those with a benign neoplasm [1,2]. From these and other works, diagnostic, predictive, and prognostic miRNA biomarkers for those who have already developed ovarian cancer have been proposed. No studies to date however are found of circulating miRNA profiling of HR women. MicroRNA profiles distinguishing women at HR for ovarian cancer from those at low risk (LR) may not only help identify those at HR but give insights into tumor-initiating pathways.

In cohorts of HR and LR patients, we have previously studied tissue protein biomarkers including CSF-1 (macrophage colony stimulating factor [6]) and ErbB4 (HER4) in ovary and fallopian tubes. We found that CSF-1 and ErbB4 expression in the adnexae correlates with both HR and BRCA carrier status [7]. Furthermore, our interest in androgen-related ovarian cancer initiation [8] led to a phase 2 biomarker seeking study demonstrating that both CSF-1 and ErbB4 are downregulated in HR patients exposed to the antiandrogen flutamide compared to HR controls. Strong evidence suggests an etiologic association between androgens and the development of ovarian cancer [9–13]. We have validated miRNA mediated downregulation of CSF-1 [14,15] and of CSF-1–related invasiveness of ovarian cancer cells [14].

The relationship between androgens and hypoxia appears to be cooperative and bidirectional. Androgen receptor (AR) and hypoxia inducible factor-1 (HIF-1α) work closely together to mediate downstream signaling. HIF-1α is a transcription factor that activates survival factor genes that function in a hypoxic environment. In many tumor types, hypoxia is associated with more aggressive disease and increased resistance to both chemotherapy and radiation therapy [16]. Androgen signaling results in HIF-1α enhanced expression and target gene activation [17–19]. In prostate cancer, androgen deprivation therapy and flutamide inhibit HIF-1α signaling [20,21]. In turn, HIF-1α helps induce AR transactivation [22]. AR signaling is active in normal ovarian aging [23,24], as it is in BRCA mutated ovarian cells [25]. Thus, ability to survive under hypoxic conditions may be one downstream effect of androgen signaling.

Our group has studied hypoxia in ovarian cancer tissues [26]. Using a hypoxia 51 gene panel [27] as the gold standard, our work validated a subset (nine genes) using a different methodology; these genes were upregulated when comparing ovarian cancer samples to normal ovaries [26]. No such study comparing serum of HR and LR populations has been found.

Collectively, there is a need to more specifically identify women at HR for ovarian cancer. Circulating miRNAs in serum are stable and may represent an important relatively noninvasive biomarker in this context. In addition, miRNA profiling between from LR and HR states may give insights in tumor initiation pathways. There is rationale for a specific focus on regulation of the androgen and its related hypoxia pathways in tumor initiation. Thus, we focused our analysis specifically on those miRNAs predicted to regulate the hypoxia and androgen pathways. We hypothesize subsets of these pathway-related circulating miRNAs may be downregulated in the HR when compared to the LR population. This pilot study is the first report to our knowledge that studies circulating miRNA profiling of HR and LR women.

Material and Methods

Patient Characteristics

The definition of HR and LR was as previously strictly defined [7]. The current HR and LR cohorts did not overlap with patients in the prior study. All five LR patients had normal fallopian tubes and ovaries on pathology in the absence of concurrent cancer. Two of four HR patients carried a deleterious BRCA mutation (one BRCA1 and other BRCA2, each predisposing to risk for ovarian cancer). Both of these patients had a personal history of breast cancer and a family history of breast and pancreatic cancer. The third HR patient had a personal and family history of breast cancer with a family history of a BRIP1 mutation (which carries a moderately increased risk for ovarian cancer). She was tested BRCA1/2 negative, but her BRIP1 status is unknown. The last HR patient had a family history of breast, pancreatic, renal, and stomach cancer and did not carry a significant germline mutation. There was no attempt to match these pilot cohorts by age. The median and range of ages are as follows: HR, 42.5 years (39-50); LR, 65 years (46-71). In order to validate our methods, we also included a small cohort of ovarian cancer patients, all of whom had high-grade serous ovarian cancer. This cohort validated prior reports [1,5,28] that miR200a/c or miR141 was overexpressed in ovarian cancer serum compared to controls (Supplementary Figure S1).

Sample Preparation

Research with deidentified frozen serum obtained from the University of Arizona Cancer Center Tumor Biorepository was carried out with University of Arizona IRB determination that this did not constitute human research. Sera from four HR patients and from five LR patients were analyzed. Serum samples were prepared by combining 15 μl of serum or conditioned media with 15 μl of HTG Biofuids lysis buffer. To each sample, 3 μl of Proteinase K was added, and the samples were then incubated for 180 minutes at 50°C. The cellular-based HTG EdgeSeq technology (HTG Molecular Diagnostics, Inc.) utilized does not require a separate extraction step for miRNA. Twenty-five (25) microliters of each sample was added to each well of a 96-well plate. Human Brain Reference RNA supplied by HTG Molecular Diagnostics, Inc. (Tucson, AZ), was added to one well at 25 ng to serve as a process control.

miRNA Assay

Samples were run on an HTG EdgeSeq Processor using the HTG EdgeSeq miRNA WT assay. Following the processor step, samples were individually barcoded (using a 16-cycle PCR to add adapters and molecular barcodes). Barcoded samples were individually purified using AMPure XP beads and quantitated using a KAPA Library Quantification kit. The library was sequenced on an Illumina MiSeq using a V3 150-cycle kit with two index reads. PhiX was spiked into the library at 5%; this spike-in control is standard for Illumina sequencing libraries. Data were returned from the sequencer in the form of demultiplexed FASTQ files, with one file per original well of the assay. The HTG EdgeSeq Parser was used to align the FASTQ

files to the probe list to collate the data. Data are provided as a data table of raw, QC raw, counts per million, and median normalized. The baseline performance characteristics were established using Human Universal Reference RNA (uRNA) across all 96 wells on three 96-well plates, with each plate processed on a different instrument.

miRNA Expression Analysis

All analyses were done using R (v3.3) statistical programming and Bioconductor packages. The miRNA raw counts were converted to counts per million and log transformed. These log-transformed counts were further normalized using median normalization. miRNA expression differences between HR and LR samples were analyzed for the log-transformed normalized expression values [29]. Differential expression between groups was estimated by Limma, a moderated *t*-statistic method (Linear Models for Microarray and RNA-Seq Data [30]). This method uses a linear model and provides inference for differential expression analysis by information borrowing from genes within a sample and replicates. Due to the low number of samples, adjustment of multiple testing does not hold any significance. Volcano plot was utilized further to identify the differences in two groups. One hundred thirty-seven miRNAs that were found to be differentially expressed between the two groups of at least 2.0-fold and with a significant *P* value of less than .05 were chosen for further investigation. Hierarchical distance-based clustering of differentially expressed miRNAs between group comparisons was done to identify correlated clusters of miRNAs that show differential pattern across two groups of patients. R libraries were utilized to generate the heat maps and all the plots.

miRNA-mRNA Target Pairs

Three independent noncoding RNA resources were downloaded and queried for miRNA-mRNA target pairs. First, predicted conserved miRNA-mRNA binding sites for mammalian genes were downloaded from TargetScan (V7.2). This is the most comprehensive up-to-date miRNA resource where predictions are ranked based on the predicted efficacy of targeting as calculated using cumulative weighted scores of the sites [31] and also by their probability of conserved targeting [32]. These sites are conserved across mammalian species and are based on conserved 8mer or 7mer sites in miRNAs. The conserved miRNA binding site may be validated or not by an experimental method. Locally created shell scripts were utilized for mapping these data to differentially expressed miRNAs found within group comparisons. Second, miRTarBase [33], a database with miRNA targeted interactions from functional studies, was analyzed. miRTarBase represents validated miRNA-mRNA interactions from >8500 published literatures by combining natural language processing and manual curation. For miRTarBase, only the interactions that were strongly supported by reporter assays and Western blots were considered. We did not include the interactions that were reported by high-throughput sequencing methods that were classified as weak interactions. Third, a smaller database of experimentally validated miRNAs from literature curation, miRecords [34], was also queried but was found to have significant overlap with the first two databases. List of genes known to be associated with Androgen Receptor Pathway was downloaded from Kegg and Reactome databases. Hypoxia-related list of genes (51) was the ones identified by Buffa et al. [27], and we added HIF1α to the list (Supplementary Table S1). miRNA interactions for this specific list of genes were summarized from noncoding resource.

qRT-PCR for miRNA Levels

We chose to validate the findings by focusing on miRNAs which were significantly downregulated in the HR compared to the LR cohort. Six miRNAs (miR-93-5p, -19a-3p, -22-3p, -106b-5p, -362-5p, and -210-3p) among the top 25 most differentially expressed in this comparison were chosen for stem-loop qRT-PCR validation [35,36]. Serum was available from four each of the LR and HR cohorts for analysis. Small RNAs were isolated using Qiagen miRNeasy Serum/Plasma kit. Primers were created for each miRNA sequence. Stem-loop qRT-PCR was performed for each miRNA, and results were normalized to 5S rRNA control. Independent experiments were performed twice for each condition. For each of the six miRNAs, the LR and HR cohorts were compared with the one-tail *t* test (Sigma plot version 12.5). Primers used for reverse transcription and PCR are described in Supplementary Table S2.

Results

Dysregulated miRNAs in HR Women

There were a total of 137 dysregulated miRNAs found between the HR and LR groups. This represented 6.6% of the 2083 miRNAs available in the platform we used in this study, or 17.1% of what is generally accepted as the 800 total circulating miRNAs found to date. Differential regulation was defined by $P < .05$ and at least two-fold change in expression. As mentioned in methods, the low number of samples was not enough to test the significance by multiple comparison testing. We utilized statistical method volcano plot that arranges genes along dimensions of biological and statistical significance as shown in Figure 1*A*. By unsupervised heat map clustering, it is clearly seen that there are three-fold more miRNAs which are downregulated than upregulated in the HR cohort when compared to the LR cohort. In fact, only 36 miRNAs were upregulated in HR samples and 101 miRNAs downregulated in HR samples when compared to LR samples (Table 1, Figure 1*B*). This observation is consistent with our hypothesis that, in the HR cohort, there would be downregulation of miRNAs that may control key pathways.

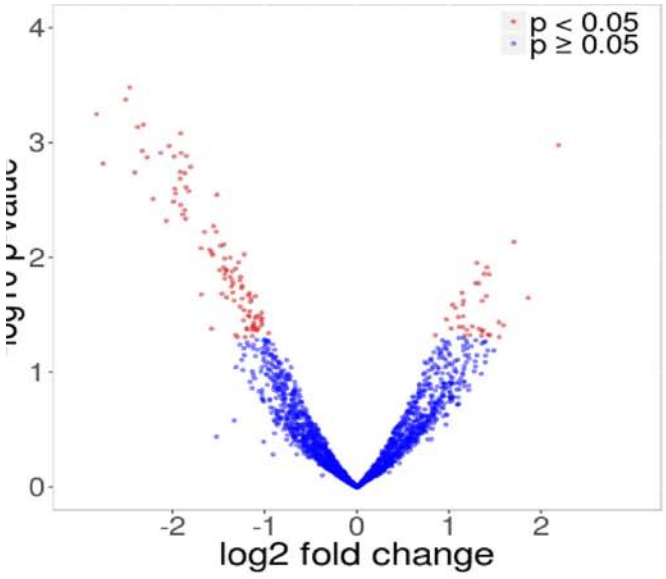
mRNA Targets of Differentially Expressed miRNAs

To characterize the functional association of these 137 miRNAs, their target mRNAs were evaluated. We computationally predicted and experimentally validated miRNA-mRNA interactions from three different independent resources. For our analysis, we chose the conserved binding sites interactions from TargetScan and interactions with strong evidence by reporter assays, Western blots, and qPCR from miRTarBase. This may not necessarily include all the individual literature that validates specific miRNAs in their function on specific genes. Analyzing conserved binding sites from TargetScan, we found 7533 genes targeted by 33 unique miRNAs that have lower expression in our HR samples and 634 number of genes targeted by only 1 miRNA that has a higher expression in HR cohort (Supplementary Table S3). The validated miRTarBase had 71 miRNAs that are downregulated from the HR samples targeting 1059 genes and 5 miRNAs that are upregulated in HR samples targeting 6 genes (Supplementary Table S3). There were 29 miRNAs from the downregulated list and 1 miRNA from the upregulated list that were common between the results from both resources.

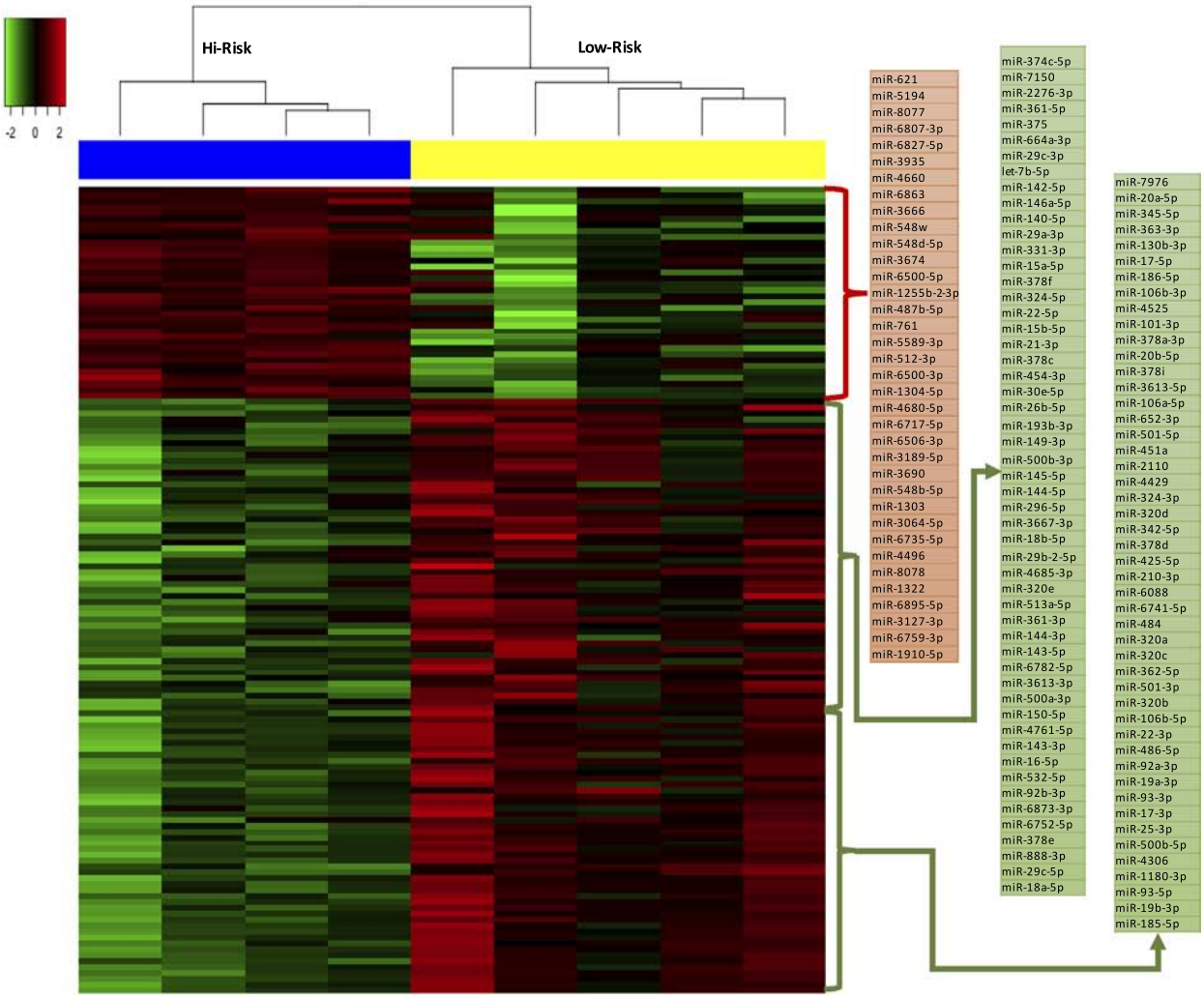
Functional Association of mRNA Targets of Dysregulated miRNAs

For both hypoxia- and androgen-focused analyses, the information generated from TargetScan (Supplementary Table S4) was further

A


p < 0.05
p ≥ 0.05
log10 p value
log2 fold change

B


Hi-Risk
Low-Risk
miR-621
miR-5194
miR-8077
miR-6807-3p
miR-6827-5p
miR-3935
miR-4660
miR-6863
miR-3666
miR-548w
miR-548d-5p
miR-3674
miR-6500-5p
miR-1255b-2-3p
miR-487b-5p
miR-761
miR-5589-3p
miR-512-3p
miR-6500-3p
miR-1304-5p
miR-4680-5p
miR-6717-5p
miR-6506-3p
miR-3189-5p
miR-3690
miR-548b-5p
miR-1303
miR-3064-5p
miR-6735-5p
miR-4496
miR-8078
miR-1322
miR-6895-5p
miR-3127-3p
miR-6759-3p
miR-1910-5p
miR-374c-5p
miR-7150
miR-2276-3p
miR-361-5p
miR-375
miR-664a-3p
miR-29c-3p
let-7b-5p
miR-142-5p
miR-146a-5p
miR-140-5p
miR-29a-3p
miR-331-3p
miR-15a-5p
miR-378f
miR-324-5p
miR-22-5p
miR-15b-5p
miR-21-3p
miR-378c
miR-454-3p
miR-30e-5p
miR-26b-5p
miR-193b-3p
miR-149-3p
miR-500b-3p
miR-145-5p
miR-144-5p
miR-296-5p
miR-3667-3p
miR-18b-5p
miR-29b-2-5p
miR-4685-3p
miR-320e
miR-513a-5p
miR-361-3p
miR-144-3p
miR-143-5p
miR-6782-5p
miR-3613-3p
miR-500a-3p
miR-150-5p
miR-4761-5p
miR-143-3p
miR-16-5p
miR-532-5p
miR-92b-3p
miR-6873-3p
miR-6752-5p
miR-378e
miR-888-3p
miR-29c-5p
miR-18a-5p
miR-7976
miR-20a-5p
miR-345-5p
miR-363-3p
miR-130b-3p
miR-17-5p
miR-186-5p
miR-106b-3p
miR-4525
miR-101-3p
miR-378a-3p
miR-20b-5p
miR-378i
miR-3613-5p
miR-106a-5p
miR-652-3p
miR-501-5p
miR-451a
miR-2110
miR-4429
miR-324-3p
miR-320d
miR-342-5p
miR-378d
miR-425-5p
miR-210-3p
miR-6088
miR-6741-5p
miR-484
miR-320a
miR-320c
miR-362-5p
miR-501-3p
miR-320b
miR-106b-5p
miR-22-3p
miR-486-5p
miR-92a-3p
miR-19a-3p
miR-93-3p
miR-17-3p
miR-25-3p
miR-500b-5p
miR-4306
miR-1180-3p
miR-93-5p
miR-19b-3p
miR-185-5p

combined with validated miRTarBase and miRecords. Our figures identify the database source for the prediction of association of differentially expressed miRNA and target gene pairs. Using this methodology, we identified many genes that are predicted to be regulated by these miRNAs, which are involved in the hypoxia (52 genes studied, Supplementary Table S1) (Figure 2, *A* and *B*) or androgen pathways (135 genes studied, Supplementary Table S1) (Figure 3, *A* and *B*). The androgen-regulated CSF-1 and ErbB4 genes are included in the androgen panel. In general, whether androgen or hypoxia genes were studied, it is clear that miRNAs which target these genes are overwhelmingly downregulated in the HR cohort when compared to the LR cohort (Figures 2 and 3). Thus, the following analyses describing pairing with differentially expressed miRNAs reflect those that are downregulated in the HR cohort.

Pairing of Hypoxia Genes by Downregulated miRNAs in HR Women

We examined the association of the 52 hypoxia genes identified with our differentially expressed miRNAs (Figure 2). Our previous work [26] initially showed that nine hypoxia-related genes are expressed at a much higher levels in ovarian tumors. In this data analysis, we found the circulating miRNAs that target eight of nine of these overexpressed genes have a much lower expression in serum from HR when compared to LR women. Specifically, the 3 most significant genes found in our prior work (VEGFA, SLC2A1, LDHA) had the highest number of pairings (among the 9 genes) with the differentially expressed miRNAs, with VEGFA having 22 miRNA pairings, SLC2A1 having 4, and LDHA having 5 miRNA pairings (Figure 2*A*). This suggests that these miRNAs could be regulating the expression of the studied genes.

To investigate further, we took the gold standard list of 52 genes that were identified as hypoxia specific by Buffa et al. and queried the differentially expressed list of miRNAs by utilizing the miRNA-mRNA target pair information from the 3 noncoding resources. Our data show that both HIF-1α and VEGF-A are hypoxia-related genes most extensively predicted to be regulated by miRNAs, which are downregulated in the HR cohort. HIF-1α had 16 miRNA pairings and VEGFA had 22 such miRNA pairings. Overall, there were 91 differentially expressed miRNA-mRNA pairings in the hypoxia analysis. The differentially expressed miRNAs had pairings with hypoxia genes with a range of 0-5 times, with miR-22-3p, 25-3p, 320a., and 374c-5p each showing pairings with 5 hypoxia genes. As expected, the known hypoxia-related miR-210 had miRNA pairings with the hypoxia genes HIF-1α, BNIP-3, and LDHA (Figure 2, *A* and *B*). This suggests an involvement of the hypoxia pathways in HR versus LR states.

Pairing of Androgen-Related Genes by Downregulated miRNAs in HR Women

We examined the association of the 135 androgen pathway–related genes with our differentially expressed miRNAs. Sixty miRNAs that target the androgen related genes were similarly found to be overwhelmingly downregulated in the HR cohort. These genes related to the androgen pathway also had a similar expression pattern where all 60 but one of the circulating miRNAs that target these genes have low expression in HR serum (Figure 3, *A* and *B*). Specifically, there were 20 individual miRNAs which each had ≥10 pairings with the androgen-related genes. Out of 60 miRNAs that target these genes, 25 were found by both TargetScan and miRTarBase, 8 were predicted by TargetScan alone, and 27 were validated only by miRTarBase. Overall, there were 429 differentially expressed miRNA-mRNA pairs in this androgen-related analysis. This indicates that there may be androgen pathway dysregulation between LR and HR states.

We identified several circulating miRNAs that were downregulated in the HR cohort which are predicted to regulate CSF-1 ($N = 6$) or ErbB4 ($N = 10$), but only one predicted miRNA for CSF-1R. Moveover, of interest to this study of differential regulation of miRNAs in LR and HR states is the finding that the androgen-regulated BRCA1 gene is associated with dysregulation of five miRNAs. These five miRNAs found downregulated in the HR cohort include miR-16-5p; 20b-5p; 15a-5p; and, importantly, 146a-5p and 361-5p. These latter two miRNAs were also predicted to regulate ErbB4.

Experimental Validation of a Small Subset of Differentially Expressed miRNAs

Six differentially expressed miRNAs were studied by qRT-PCR to compliment the RNAseq methodology utilized. Out of the six miRNAs, miR-19a-3p and miR-22-3p (Supplementary Figure S2) were both significantly downregulated in the HR cohort ($P = .002$, and .005, respectively). Another three miRNAs, miR-93-5p, miR-362-5p, and miR-210-3p, showed a trend towards significance for downregulation in the HR cohort ($P = .053$, .0578, and .050, respectively). The remaining miR-106b-5p comparison was not significant for downregulation in the HR cohort ($P = .075$). While our validation is of a tiny subset of the miRNAs found to be dysregulated by RNAseq, these positive findings are encouraging especially in light of the small number of samples. The five of six miRNAs (miR-93-5p, -19a-3p, -22-3p, -362-5p, and -210-3p) which found significance or near significance by qRT-PCR each target one of four genes of interest to us (CSF-1, CSF-1R, ErbB4, or HIF-1α).

Discussion

MicroRNA profiles distinguishing women at HR for ovarian cancer from those at LR may not only help identify those at HR but give insights into tumor-initiating pathways. No studies to date are found of circulating miRNA profiling of LR and HR women. Our data show that dysregulation of circulating miRNA appears to be widespread between LR and HR states. We identify a total of 137 dysregulated circulating miRNAs, which represent approximately 17% of the known total circulating miRNAs to date. The large majority (74%) or 101 miRNAs were found to be downregulated in HR versus LR states.

We focused our analysis on differentially expressed circulating miRNAs which may impact the hypoxia or androgen pathways. Circulating miRNA profiling shows many dysregulated profiles among those acclimated compared to those not acclimated to hypoxic

Figure 1. Differentially expressed miRNAs between HR and LR samples. (A) Volcano plot shows the horizontal axis as the fold change between the two groups on a log scale, and vertical axis represents the *P* value for test of differences between samples. (B) Hierarchical cluster of miRNA expression profiles ($N = 137$ miRNAs) of HR and LR serum showing correlation among the differentially expressed downregulated miRNAs versus those that are upregulated.

Table 1. The Table Shows Differential Expression in Fold Change with Significant *P* Values in HR Samples When Compared with LR Samples

miRNA	LogFC	*P*_Value
miR-621	2.189	.001
miR-5194	1.8595	.023
miR-8077	1.7055	.007
miR-6807-3p	1.5945	.039
miR-6827-5p	1.5465	.049
miR-3935	1.5405	.037
miR-4660	1.4425	.048
miR-6863	1.4325	.014
miR-3666	1.4265	.047
miR-548w	1.4145	.012
miR-548d-5p	1.4025	.022
miR-3674	1.3855	.014
miR-6500-5p	1.369	.043
miR-1255b-2-3p	1.3595	.024
miR-487b-5p	1.3595	.044
miR-761	1.3555	.048
miR-5589-3p	1.327	.042
miR-512-3p	1.3215	.017
miR-6500-3p	1.3045	.011
miR-1304-5p	1.293	.017
miR-4680-5p	1.2555	.040
miR-6717-5p	1.2245	.048
miR-6506-3p	1.194	.043
miR-3189-5p	1.1695	.048
miR-3690	1.1615	.024
miR-548b-5p	1.155	.033
miR-1303	1.1505	.040
miR-3064-5p	1.1485	.026
miR-6735-5p	1.142	.020
miR-4496	1.1105	.040
miR-8078	1.1015	.033
miR-1322	1.07	.028
miR-6895-5p	1.039	.026
miR-3127-3p	1.0235	.042
miR-6759-3p	0.9735	.035
miR-1910-5p	0.8555	.048
miR-374c-5p	−0.9565	.046
miR-7150	−1.0235	.034
miR-2276-3p	−1.029	.037
miR-361-5p	−1.0305	.039
miR-375	−1.0325	.031
miR-664a-3p	−1.048	.035
miR-29c-3p	−1.0575	.032
let-7b-5p	−1.067	.041
miR-142-5p	−1.0855	.043
miR-146a-5p	−1.0885	.043
miR-140-5p	−1.0935	.033
miR-29a-3p	−1.1	.022
miR-331-3p	−1.105	.034
miR-15a-5p	−1.112	.039
miR-378f	−1.1185	.037
miR-324-5p	−1.124	.041
miR-22-5p	−1.1315	.040
miR-15b-5p	−1.134	.043
miR-21-3p	−1.1365	.049
miR-378c	−1.1485	.023
miR-454-3p	−1.1545	.024
miR-30e-5p	−1.161	.030
miR-26b-5p	−1.167	.033
miR-193b-3p	−1.1675	.021
miR-149-3p	−1.176	.022
miR-500b-3p	−1.181	.032
miR-145-5p	−1.1895	.042
miR-144-5p	−1.1915	.041
miR-296-5p	−1.2125	.050
miR-3667-3p	−1.2215	.009
miR-18b-5p	−1.225	.029
miR-29b-2-5p	−1.23	.035
miR-4685-3p	−1.2435	.018
miR-320e	−1.244	.023
miR-513a-5p	−1.2495	.018
miR-361-3p	−1.2605	.015
miR-144-3p	−1.2635	.027
miR-143-5p	−1.266	.016
miR-6782-5p	−1.2795	.011
miR-3613-3p	−1.2935	.050
miR-500a-3p	−1.3105	.047
miR-150-5p	−1.314	.013
miR-4761-5p	−1.3315	.024
miR-143-3p	−1.339	.017
miR-16-5p	−1.3455	.020
miR-532-5p	−1.3515	.016
miR-92b-3p	−1.352	.013
miR-6873-3p	−1.363	.018
miR-6752-5p	−1.3725	.015
miR-378e	−1.374	.010
miR-888-3p	−1.416	.022
miR-29c-5p	−1.417	.013
miR-18a-5p	−1.42	.013
miR-7976	−1.4335	.014
miR-20a-5p	−1.4365	.015
miR-345-5p	−1.439	.010
miR-363-3p	−1.443	.012
miR-130b-3p	−1.4445	.008
miR-17-5p	−1.465	.021
miR-186-5p	−1.4805	.008
miR-106b-3p	−1.4865	.013
miR-4525	−1.5175	.003
miR-101-3p	−1.5255	.006
miR-378a-3p	−1.557	.005
miR-20b-5p	−1.561	.010
miR-378i	−1.575	.009
miR-3613-5p	−1.5755	.042
miR-106a-5p	−1.593	.009
miR-652-3p	−1.6495	.006
miR-501-5p	−1.6825	.021
miR-451a	−1.69	.008
miR-2110	−1.806	.002
miR-4429	−1.827	.003
miR-324-3p	−1.85	.001
miR-320d	−1.856	.002
miR-342-5p	−1.8585	.005
miR-378d	−1.8675	.002
miR-425-5p	−1.8705	.004
miR-210-3p	−1.8885	.004
miR-6088	−1.906	.001
miR-6741-5p	−1.91	.001
miR-484	−1.9115	.003
miR-320a	−1.9165	.002
miR-320c	−1.9175	.002
miR-362-5p	−1.9665	.003
miR-501-3p	−1.9745	.003
miR-320b	−1.98	.001
miR-106b-5p	−1.988	.003
miR-22-3p	−2.036	.001
miR-486-5p	−2.0675	.005
miR-92a-3p	−2.131	.001
miR-19a-3p	−2.2125	.003
miR-93-3p	−2.276	.001
miR-17-3p	−2.315	.001
miR-25-3p	−2.327	.001
miR-500b-5p	−2.379	.001
miR-4306	−2.409	.002
miR-1180-3p	−2.4645	.000
miR-93-5p	−2.5095	.000
miR-19b-3p	−2.752	.002
miR-185-5p	−2.825	.001

(continued on next page)

conditions [37,38]. The four major signaling pathways disrupted by high altitude include the HIF-1, cAMP, MAPK, and NF-kappa B pathways [38]. miRNA signature of hypoxia of cells *in vitro* [39], identifies several miRNAs as being induced in response to hypoxia.

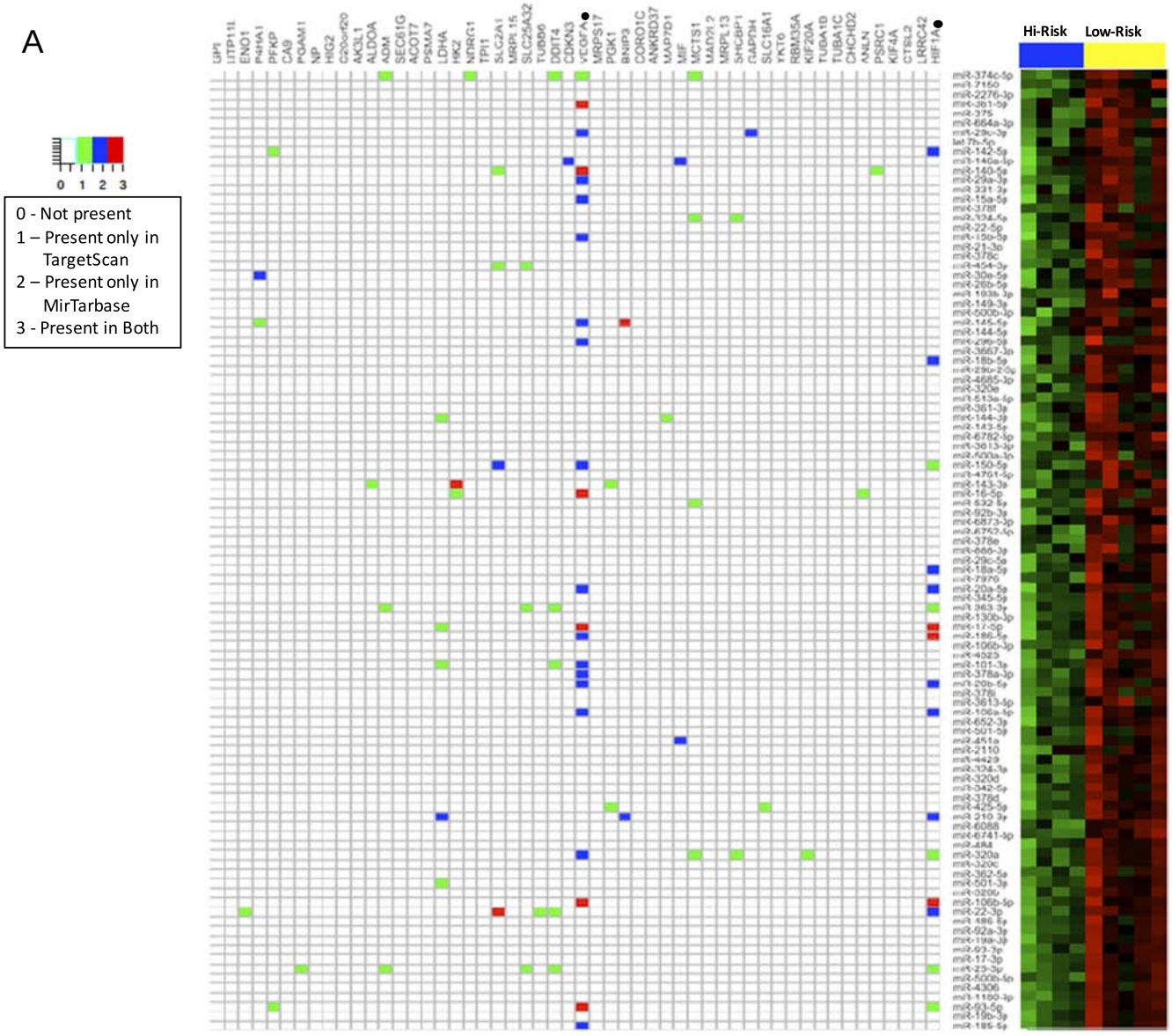


Figure 2. Heat map of 137 miRNAs and their paired interactions with 52 hypoxia genes. The expression cluster shows upregulated miRNAs in red and downregulated miRNAs in green in HR and LR samples. (A) The 101 miRNAs that are downregulated in HR samples and interaction of 39 miRNAs with hypoxia genes. (B) The 36 miRNAs that are upregulated in HR samples and interactions of the 1 miRNA found with hypoxia genes.

However, no prior studies of miRNA profiling of ovaries or fallopian tubes of HR versus LR women were found.

We have previously validated a nine-gene hypoxia-regulated panel in ovarian cancer [26]. The most significant three hypoxia-related genes associated with ovarian cancer tissue (compared to normal control tissue) included SLC2A1, LDHA, and VEGFA. Our results show that circulating miRNAs which regulate those three genes were significantly downregulated in serum from HR patients compared to those who are LR. Using a 52-gene hypoxia panel as a gold standard [27], we found that other notable genes targeted by miRNAs downregulated in the HR cohort include HIF-1α. Among conserved and largely validated miRNAs, all 39 miRNAs, with the exception of 1 miRNA (miR3064-5p), were downregulated in the HR cohort. This suggests that the hypoxia pathway is active in HR states.

Circulating miRNAs related to androgen dysregulation are largely found in men with prostate cancer [40]. miRNAs found to be circulating in men with castrate-resistant prostate cancer overlap with four downregulated circulating miRNAs in our HR cohort. These include miR-20a-5p, 20b-5p, 375, and 210-3p. Among conserved and largely validated miRNAs, all 60 miRNAs related to the androgen signaling pathway, with the exception of 1 miRNA (miR3064-5p), were downregulated in the HR cohort. In addition, the magnitude of the predicted miRNA:mRNA pairings (N = 429) in the androgen pathway is impressive. The finding that five miRNAs which are predicted to target BRCA1 are downregulated in the HR cohort is novel. In general, miRNAs downregulate the targets to which they bind [41,42]; however, there are exceptions such as miR-335, which has been described to positively regulate BRCA1 [43]. BRCA1 gene expression can be regulated epigenetically, such as by BRCA1 promoter hypermethylation, and these miRNA findings suggest another epigenetic mechanism for BRCA1 dysregulation in the HR cohort. Collectively, this suggests that the androgen pathway may be very active in the HR versus LR states.

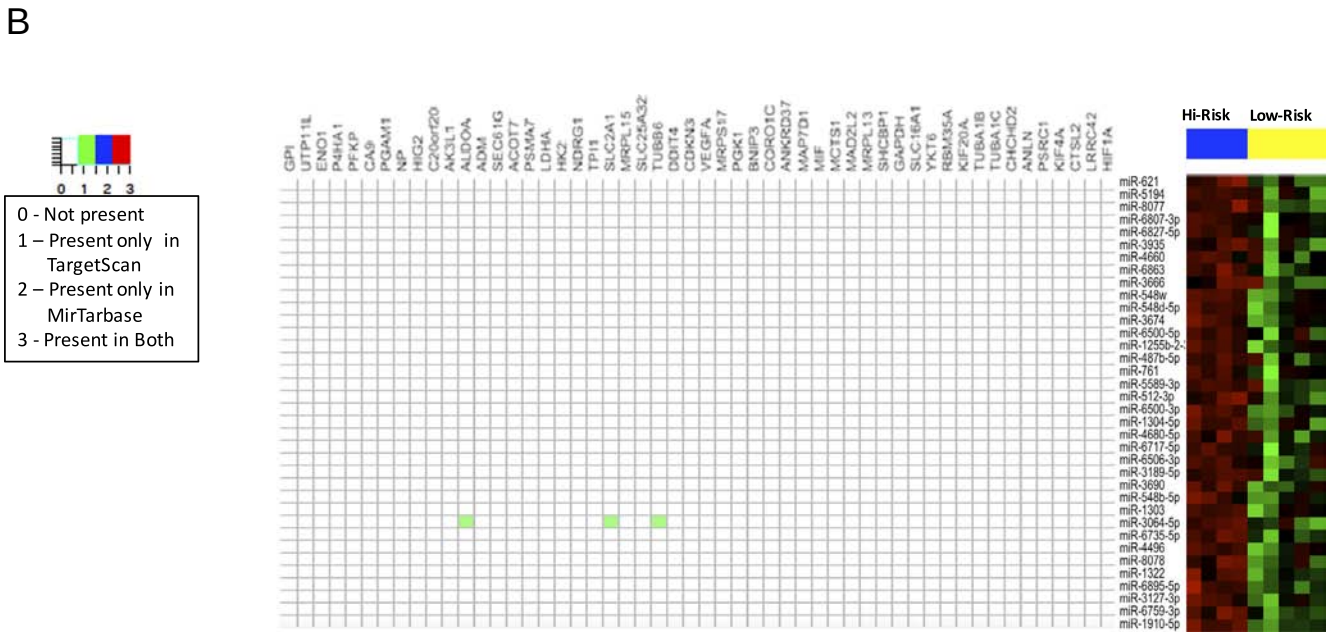


Figure 2. (*continued*)

CSF-1 and ErbB4 are a few of the many proteins known to be regulated by the androgen signaling pathway [8]. In addition to our *in vitro* validation of miR128, miR152, miR130a, and miR301a [14,15] and that of miR-26a [44] as regulating CSF-1, our current results show that six other miRNAs predicted to regulate CSF-1, including miR-130b and miR-26b-5p, are downregulated in serum of HR compared to LR women.

ErbB4 is increasingly known to be regulated in tissue by miRNAs. We found one circulating miRNA which was downregulated in the HR cohort which has been recognized in tissue to regulate ErbB4 (miR-140-5p) [45] and another (miR-193b-3p) which was related to miR-193a-3p found to regulate ErbB4 in tissues [46].

Our findings suggest that several circulating miRNAs each predicted to regulate CSF-1 and ErbB4, but only one for CSF-1R, are downregulated during in the HR state. These findings are in agreement with the significant overexpression of CSF-1 and ErbB4 proteins, but not of CSF-1R, found in HR compared to LR ovarian and fallopian tube tissues [7]. There is little work found on circulating miRNAs which regulate either CSF-1 or ErbB4.

Because we were curious as to the role of the smaller group (N = 36) of miRNAs found to be upregulated in the HR compared to the LR cohort and our analysis had focused on the conserved miRNA binding sites, we investigated the differentially expressed miRNAs in poorly conserved miRNA-mRNA target pairs from TargetScan. We found 32 miRNAs that are upregulated in the HR cohort with 17,242 gene targets and 36 miRNAs that are downregulated in the HR cohort with 18,336 targets. We found a much larger interaction for hypoxia- (75 miRs targeting 44 genes) and androgen-related genes (78 miRs targeting 132 genes) for both up- and downregulated miRNAs in the HR cohort. We did note a very large overlap between these nonconserved miRNAs and those found to be overexpressed in the HR cohort. These interactions with no conserved binding sites or confidence to support were not investigated further.

The only conserved predicted miRNA by TargetScan that is upregulated in HR cohort is predicted to target 627 genes including those in the hypoxiap and androgen-related pathways; thus, its putative activity is widespread.

The miRNAs that are downregulated in the HR cohort target genes (N = 7423) that are enriched in multiple cellular processes beyond hypoxia and androgen genes. These include genes in TGF-beta, Hippo, MAPK, cAMP, Wnt, and phosphatidylinositol signaling system. The other overrepresented functions are axon guidance, ubiquitin-mediated proteolysis, adherens junction, and regulation of cytoskeleton. In the miRTarBase, the downregulated miRNAs are found to interact with 983 other genes excluding hypoxia and androgen genes. Pathway analysis yields some of the same signaling and cell-cell communication pathways being targeted as the ones we found with TargetScan gene targets.

We propose that miRNA dysregulation may play critical roles in the early development of ovarian cancer. Analysis of specific miRNAs can give insight into pathways that are dysregulated in HR versus LR states. Our pilot study shows that circulating miRNA dysregulation is widespread in this context. It is possible that via miRNA dysregulation, hypoxia-related genes as well as those in the androgen signaling pathway may be upregulated during the early stages of ovarian cancer initiation. What is always an unknown in a study of circulating miRNAs is the tissue or cellular origin of these circulating miRNAs. Thus, a clear assumption cannot be made that cells from the ovaries or fallopian tubes from the HR or LR women are the source of these differentially expressed miRNAs. miRNA profiling of tissues from HR women is necessary to study the cellular sources of these circulating miRNAs to lend insight into biological mechanisms.

Our report is based on predictive methodology but includes three databases for target prediction. A major weakness is the very small numbers of clinical samples studied. Experimental validation with qRT-PCR was performed of six miRNAs that were significantly downregulated in the HR cohort, with the finding of significance or near significance in five of six miRNAs chosen for study. This is encouraging; however, weakness remains as to the need for further experimental validation of miRNA dysregulation between the two

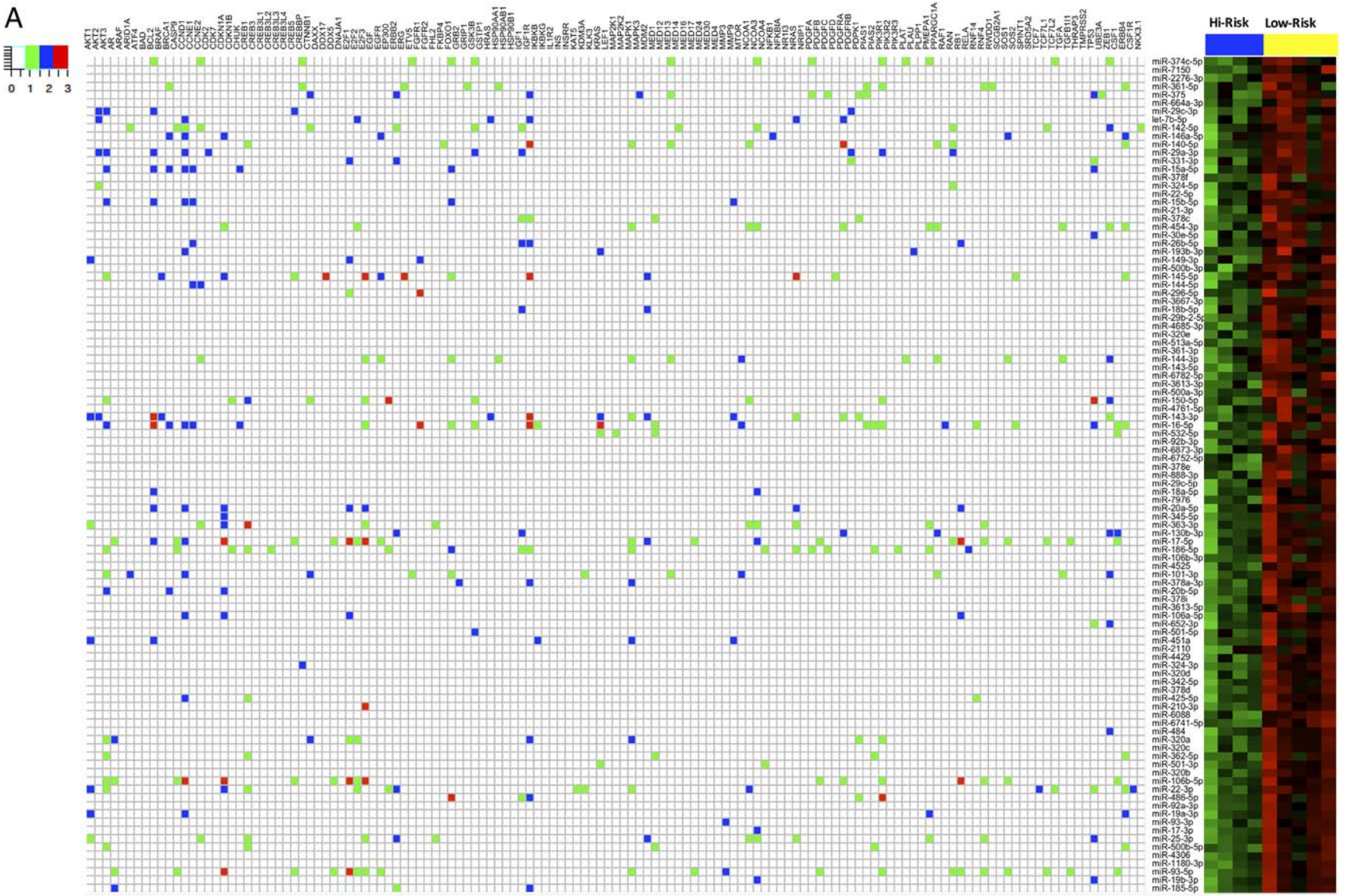


Figure 3. Heat map of 137 miRNAs and their paired interactions with 135 androgen-related genes. The expression cluster shows upregulated miRNAs in red and downregulated miRNAs in green in HR and LR samples. (A) The 101 miRNAs that are downregulated in HR samples and interaction of 60 miRNAs with androgen genes. (B) The 36 miRNAs that are overexpressed in HR samples and interactions of the 1 miRNA found with androgen-related genes.

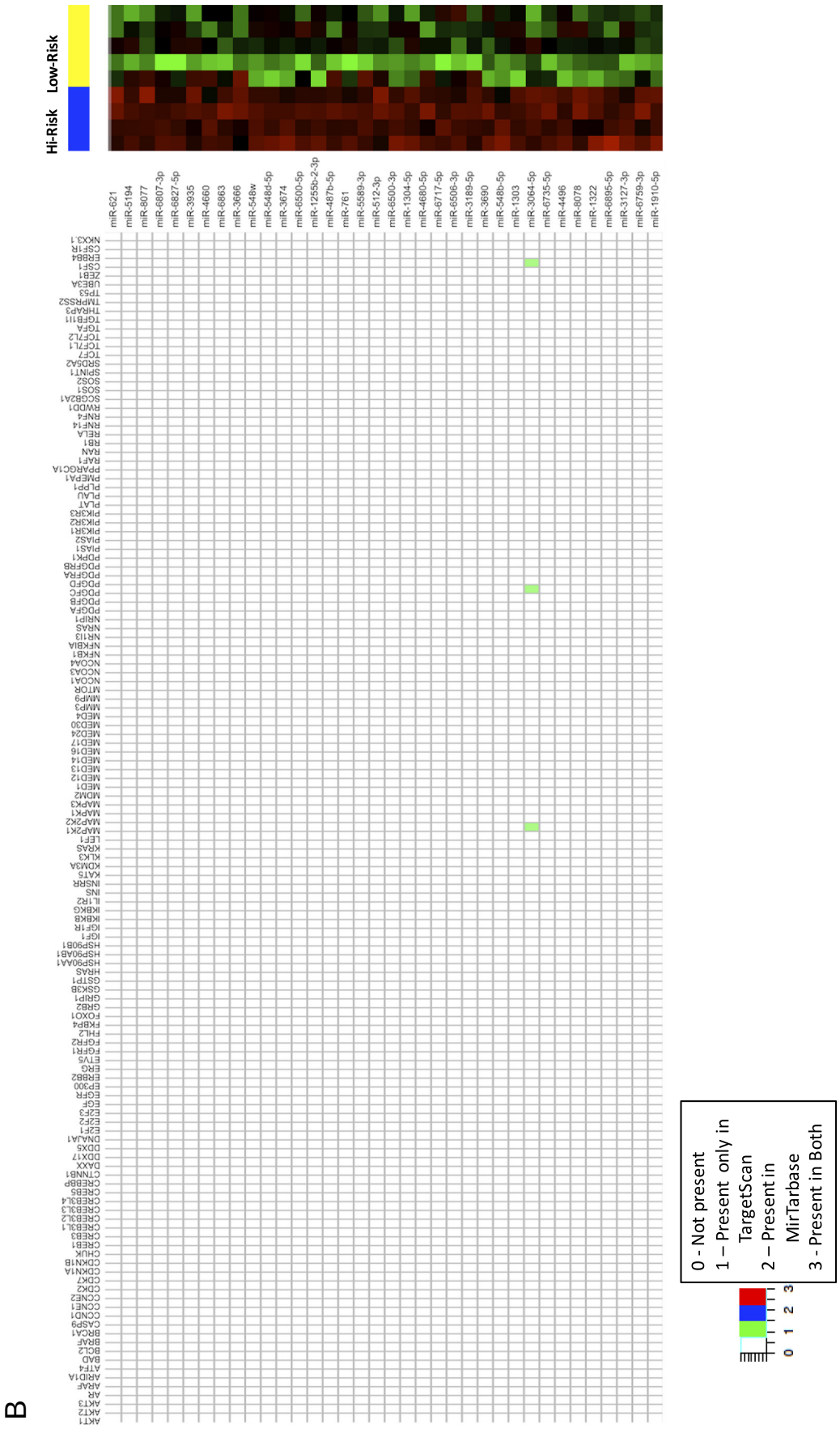


Figure 3. (*continued*)

cohorts for a larger number of miRNAs. From a biostatistical point of view, while the significance of the RNAseq findings reflect at least two-fold difference and a P value < .05, the adjusted P values were not significant, reflecting the small number of samples. This pilot study clearly needs to be validated by larger studies. There are known considerations for discrepancy in studies which focus on circulating miRNAs [47]. These include the methodology (platform), their sensitivity and specificity, power of the study, the bioinformatics analysis and the stringency, and specificity of the biomarker findings. These factors impact on the reproducibility of the results.

Conclusion

To our knowledge, our preliminary report is the first to study circulating miRNAs in serum from HR and LR women. The data suggest a widespread alteration of circulating miRNA profiles between HR and LR women. Analysis of specific circulating miRNAs in our pilot study, which is preliminary and requires validation in a larger study, gives potential insight into pathways that are dysregulated between LR and HR states. These analyses suggest dysregulation of hypoxia and its related androgen pathways between LR and HR states.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tranon.2019.01.006.

Competing Interests

The authors declare that they have no competing interests.

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