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Research Report

Transcriptomic analysis of patient plasma reveals circulating miR200c as a potential biomarker for high-grade serous ovarian cancer

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ABSTRACT

Background: High-grade serous tubo-ovarian cancer (HGSC) is the most common histological subtype of epithelial ovarian cancer, and highly lethal. Currently there is no effective screening test and prognosis is poor as the majority of cases are diagnosed at the advanced stage. Cell free RNAs including microRNAs (miRNAs) are dysregulated in ovarian cancer tissue and are detectable in the circulation. This study aimed to determine whether circulating cell free miRNAs may be potential biomarkers for the detection of HGSC.

Methods: Plasma was collected from women with HGSC (Grade 3, n = 24), and benign ovarian masses (n = 24). RNA was extracted from patient plasma and subjected to miRNA targeted next generation sequencing (NGS). A subsequent validation cohort was assessed using plasma collected from women with HGSC (n = 14) and controls (with a benign ovarian mass; n = 15). RNA was extracted and assessed using quantitative RT-PCR.

Results: Differential gene expression (DGE) of the NGS data revealed a significant increase in the miRNA, miR200c, in the circulation of women with HGSC (p less than 0.05) compared to controls. In the validation cohort miR200c expression by qPCR was found to also be increased in the circulation of women with HGSC compared to controls (p = 0.0023).

Conclusions: Circulating miR200c may be a promising candidate biomarker for the detection of HGSC. Further larger cohort studies exploring earlier stages are needed to determine whether circulating miR200c may be a sensitive and specific biomarker of tubo-ovarian cancer.

1. Background

Ovarian cancer is the fifth most lethal cancer in women, accounting for the majority of gynaecological cancer deaths (Siegel et al., 2012). In 2018 there were an estimated 295,000 ovarian cancer diagnoses and 184,000 deaths worldwide (Bray et al., 2018). High-grade serous tubo-ovarian cancer (HGSC) is the most common histological subtype. Women present with advanced stage disease (FIGO stages 3 and 4) and less than 50% are living 5 years after diagnosis (Anuradha et al., 2014). However the 5-year survival rate for early stage disease (FIGO stage 1) is over 80% (Ahmed et al., 1996). There is no evidence to support routine population-based screening for ovarian cancer. There is no test, including pelvic examination or ultrasound, serum CA125 or other tumour markers shown to reduce mortality (Jacobs et al., 2016; Woodward et al., 2007). Because advanced HGSC is mostly incurable, there is urgent need for a sensitive test to detect disease at an early asymptomatic stage when treatment is possible.

MicroRNAs (miRNAs) are small noncoding RNA, ~19–25 nucleotides in length, that regulate gene expression by binding to the 3′ untranslated region of target messenger RNAs (mRNAs), inhibiting

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translation or inducing mRNA degradation (Cullen, 2004). MiRNAs play key roles in cell differentiation, growth and apoptosis and aberrant miRNA expression has been observed in human malignancies (Harris, 2002). Thus, miRNAs may act as oncogenes or tumour suppressor genes, depending on their targets. Microarray technologies have identified differential miRNA expression in normal and tumour tissues including plasma, and specific miRNA expression signatures associated with response to systemic treatment and prognosis (Prahm et al., 2018). Unfortunately PCR-based miRNA microarrays do not enable detection of novel miRNAs or prediction of downstream target genes and signalling pathways (Zuberi et al., 2015; Srivastava et al., 2017). The discovery that circulating microRNAs in the blood may reflect gene expression in a distant tumor, has enhanced enthusiasm to measure circulating miRNAs as biomarkers for cancer detection and prognosis.

Thus, the objective of the current study was to investigate circulating plasma miRNAs in a cohort of women with HGSC and benign controls using next generation RNA sequencing to identify potential miRNA candidates that could be used to screen for HGSC in future validation studies.

2. Methods

2.1. Study participants

Participants were recruited from the St John of God Hospital, Western Australia Gynecologic Oncology Biospecimen Bank (Human Research Ethics Committee Approval Reference 360) between 2013 and 2016. The St John of God Health Care Ethics Committee approved the study protocol (Reference 1163).Written informed consent was obtained prior to inclusion.

Discovery cohort: Cases of HGSC (n = 24) were ascertained from the Western Australia Gynecologic Oncology Biospecimen Bank. All cases were reviewed at a Gynecologic Oncology Tumor Board meeting, confirming the histopathological diagnosis and FIGO stage. Controls (n = 24) were aged matched women with benign ovarian masses. ***Validation cohort***: HGSC cases (n = 14) were confirmed as described above and controls (n = 15) were aged matched with benign ovarian masses. Patients with a history of non-HGSC tubo-ovarian, other gynecologic, previous or recurrent cancer were excluded.

2.2. Sample collection

Peripheral whole blood samples (2.5 ml) were collected, prior to surgery in EDTA tubes. Tubes were centrifuged for 5 mins at 1000g. Plasma then transferred into 1 ml aliquots and stored at −80 °C until miRNA extraction.

2.3. Extraction and preparation of miRNA from patient plasma

miRNA was extracted from plasma samples using the QIAmp Circulating Nucleic Acid Kit (Qiagen, Valencia, CA). Samples were then purified using the Purification of Circulating RNA step on the QIAVac 24 plus vacuum manifold according to manufacturer's instructions. Genomic DNA was removed using the QIAGEN RNase-Free DNase Set, and miRNA was then applied to the RNeasy MinElute Cleanup kit. RNA was eluted into RNAse free water and stored at −80 °C. The concentration of total RNA was quantified by NanoDrop 1000 (Nanodrop).

All samples were extracted from equal starting volumes: 1 ml of plasma was used for miRNA extraction for the Next Generation Sequencing discovery step and 100ul of plasma was used to extract miRNA for the quantitative PCR validation step.

2.4. Library preparation

Libraries were prepared and samples sequenced at Australian Genome Research Facility (AGRF) in Melbourne using a HiSeq 2500 Illumina sequencer with 50 nt single-end reads. NEBNext Small RNA Library Prep Set according to manufacturer's instructions with pippin prep size selection range 45–160 bp (RNA if 18–33 nt) was used.

2.5. Targeted next generation sequencing to identify miRNAs

Data were analysed with bcbio-nextgen (https://github.com/bcbio/bcbio-nextgen) using piDNA to detect the adapter, and cutadapt was used to remove the adapter. We used STAR/bowtie to align against the genome, and seqcluster to detect small RNA transcripts. Detection of miRNAs was performed using miraligner tool with miRBase as the reference miRNA database. Detection of tRNA profiles were detected using the tdrmapper tool. Discovery of miRNAs was identified using mirdeep2. FastQC was used for QC metrics and multiqc for reporting. DESeq2 was then used for the Digital Gene Expression (DGE) analysis.

2.6. Differential gene expression

Using the miRNA counts matrix, counts_mirna.tsv we then used DESeq2 (Prahm et al., 2018) to perform a statistical analysis of the counts for each miRNA. Versions: R: 3.5.1 DESeq2 version: 1.20.0 Complex Heatmap: 1.18.1 limma: 3.36.5 Glimma: 1.8.2. MDS and Glimma Plots were created using limma (Zuberi et al., 2015) and Glimma (Srivastava et al., 2017) packages respectively. Heatmap was generated through the package Complex Heatmap (Pendlebury et al., 2017).

2.7. Sample omission from downstream analysis

Two patient samples were removed following sequencing since the samples were incorrectly assigned. Using the array Quality Metrics package (v 3.36.0) (Harris, 2002) we noted some samples demonstrated properties of outliers from their expression profiles. One patient sample showed a significant difference between other samples on its distance array test and was subsequently removed from the analysis.

2.8. Pathway analysis

Ingenuity Pathway Analysis (IPA; Ingenuity, Redwood City, CA) was performed to identify canonical pathways and gene networks related to ovarian cancer and plasma serum pathways to categorize differentially expressed genes in specific diseases and functions. Pathway networks were generated to identify the most relevant molecules regulated by the differentially expressed miRNAs.

2.9. Validation of plasma levels of miR200c using quantitative RT-PCR

miRNA was isolated from plasma aliquots (100ul) using the MagMax mirVANA RNA isolation kit according to manufacturer's instructions. miRNA cDNA was synthesised using the TaqMan Advanced miRNA cDNA Synthesis Kit, with 2ul input per sample as per manufacturer's instructions.

Specific TaqMan miRNA Advanced Assays were used for the quantification of the miRNAs hsa-miR-200c-3p (478351_mir), hsa-miR-191-5p (477952_mir) as the control reference housekeeping miRNA. The TaqMan Fast Advanced Master Mix was also used for optimal assessment of expression according to manufacturer's instructions.

Following cDNA generation qPCR was performed in duplicate, with multiple negative controls, on a CFX 384 (BioRad, Foster CA) with the following cycling conditions: 95C for 10mins, and 40 cycles of 95C for 15 sec, 60C for 1 min. Fold changes in expression were determined by the comparative CT method normalized against the mean expression of the reference housekeeping miRNA.

3. Results

3.1. Clinical characteristics

Clinical characteristics for the HGSC cases and controls are presented in Table 1 (*Discovery:* NGS miRNA analysis) and Table 2 (*Validation:* qRT-PCR analysis).

3.2. Assessment of circulating miRNA in plasma from women with HGSC

We used targeted next generation sequencing to examine the expression of miRNAs in the circulation to identify new candidate blood-based biomarkers for HGSC (Fig. 1). Multidimensional scaling (MDS) analysis (Fig. 1A) and Heatmap cluster analysis (Fig. 1B) demonstrated a weak correlation between the two groups across many miRNAs, suggesting very few miRNAs were differentially expressed.

We found increased expression of one miRNA has-miR-200c-3p (Fig. 1C), with a false discovery rate (FDR) for expression changes less than 0.05, and a strong significance with an FDR value of 0.0014 in plasma in cases with HGSC compared to controls with a logFC of +1.41.

3.3. Validation of miR200c expression in plasma from women with HGSC

In a separate validation cohort (n = 16 benign controls and n = 14 HGSC) expression of miR200c in the plasma of women with HGSC and benign controls was assessed. miR200c was increased in the plasma of women with HGSC compared to benign controls (Fig. 1D; p = 0.0023).

3.4. Functional canonical and disease pathway analysis

Possible molecular pathways underlying HGSC were examined using IPA, a manually curated database that identifies over-represented biological processes in a given dataset and calculates a significance score for each result using the right tailed Fisher's test. The top enriched categories of canonical pathways are listed in Table 3. In addition to canonical pathways, differentially expressed genes were categorized to related plasma serum (Fig. 2A) and ovarian cancer pathways (Fig. 2B) to consider functional networks for miR200c-3p. Assessment of the plasma/serum pathways identified relationships for miR220c-3p (and other miRNAs with the seed sequence AAUACUG) with enzymes; (PLCG1), phosphatase (PTPN13), translation regulator (AGO2), as well as myristoylated alanine-rich C-kinase substrate (MARCKS), a major substrate of protein kinase C that plays a key role in oncogenesis, and the actin organizing formin homology 2 domain 1 (FHOD1). Analysis of pathways also identified weaker relationships with vimentin (VIM). Analysis of ovarian cancer pathways revealed miR200c-3p is associated with the regulation of growth factors (VEGFA, PK3CA, PRKACB, PRKAR1A, PRKAR2B, RPS6KB1), the kinase RAP1B, the receptor EDNRA and the signalling factor WNT16 (Fig. 2B and Table 3).

Table 1
Clinical characteristics of HGSC cases and benign controls from the discovery cohort.

	Case HGSC (n = 24)	Benign matched control (n = 24)
Age median (IQR)	60.25 (55.2–69)	60.6(55–69.1)
Benign cystadenoma n (%)	0	22 (91.7)
Benign fibroma (n, %)	0	2 (8.3)
Stage		
3A	2	0
3B	3	0
3C	18	0
4	1	0

Table 2
Clinical characteristics of HGSC cases and benign controls from the validation cohort.

	Case HGSC (n = 14)	Benign matched control (n = 15)
Age median (IQR)	62.9 (56.2–71.2)	63.0 (55.6–71.2)
Benign cystadenoma n (%)	0	14 (87.5)
Benign fibroma (n, %)	0	2 (12.5)
Stage		
3A	2	0
3B	2	0
3C	9	0
4B	1	0

3.5. Validation of molecular pathways regulated by miR200c in plasma from women with HGSC

In a separate validation cohort, we aimed to assess the expression of genes of interest by IPA (*VIM, AGO2, PTPN13, MARCKS, PLCG1*) in serum/plasma studies in the plasma of women with HGSC and benign controls. Unfortunately, either the expression of these genes identified through IPA, that we subsequently selected for validation with qPCR, were below detectable levels in plasma from the validation cohort, or the input of RNA in the PCR reaction was too low for robust PCR (Cqs below threshold 35 cycles; data not shown). Thus, we were unable to reliably measure the associated genes identified with pathway analysis in plasma.

4. Discussion

Despite advances in our understanding of many human malignancies in recent decades, mortality rates for ovarian cancer patients have remained constant. HGSC is the predominant subtype of tubo-ovarian cancer and accounts for the majority of ovarian cancer deaths. Early diagnosis of HGSC is dependent on the development of robust clinical biomarkers to enhance detection of the disease at an early, curable stage and discrimination between benign and malignant tumours. Unfortunately, there is still no effective screening approach for HGSC.

MicroRNAs (miRNAs) have recently demonstrated promise in the diagnosis of other types of cancer (Poel et al., 2018; Montazerian et al., 2018; Otandault et al., 2019) owing to their stability and relative ease of detection in blood samples, allowing minimally invasive, repeated sampling. Importantly miRNAs are aberrantly expressed in a number of cancers (Pendlebury et al., 2017; Deb et al., 2018; Parizadeh et al., 2019), including HGSC (Liu et al., 2019), where they have been shown to accelerate tumor migration and invasion (Liu et al., 2019). However reduced expression of miRNAs can have the opposite effect, miR182 was associated with higher expression of discoidin domain receptor 2 (DDR2), a tumour promoter, in women with HGSC (Ramalho et al., 2019) and lower circulating levels of miR182 had a worse prognosis (Ramalho et al., 2019) suggesting that miRNAs both positively and negatively regulate tumour growth and disease progression.

Previously we identified altered expression of the miR200 family in the circulation from women with epithelial tubo-ovarian cancer (Pendlebury et al., 2017). Importantly this increase in expression correlated with stage of disease and reflected the relative tissue expression (Pendlebury et al., 2017). The miR200 family have been implicated as playing a key role in initiating cancer development and invasion (Pal et al., 2015). The miR200 family target two transcription factors, ZEB1 and ZEB2, which regulate E-cadherin expression and cellular polarity (Bendoraite et al., 2010). Increased ovarian tissue expression of miR200a and 200c have previously been shown to be associated with reduced overall survival in patients with epithelial tubo-ovarian cancers, possibly attributed to alterations in response to

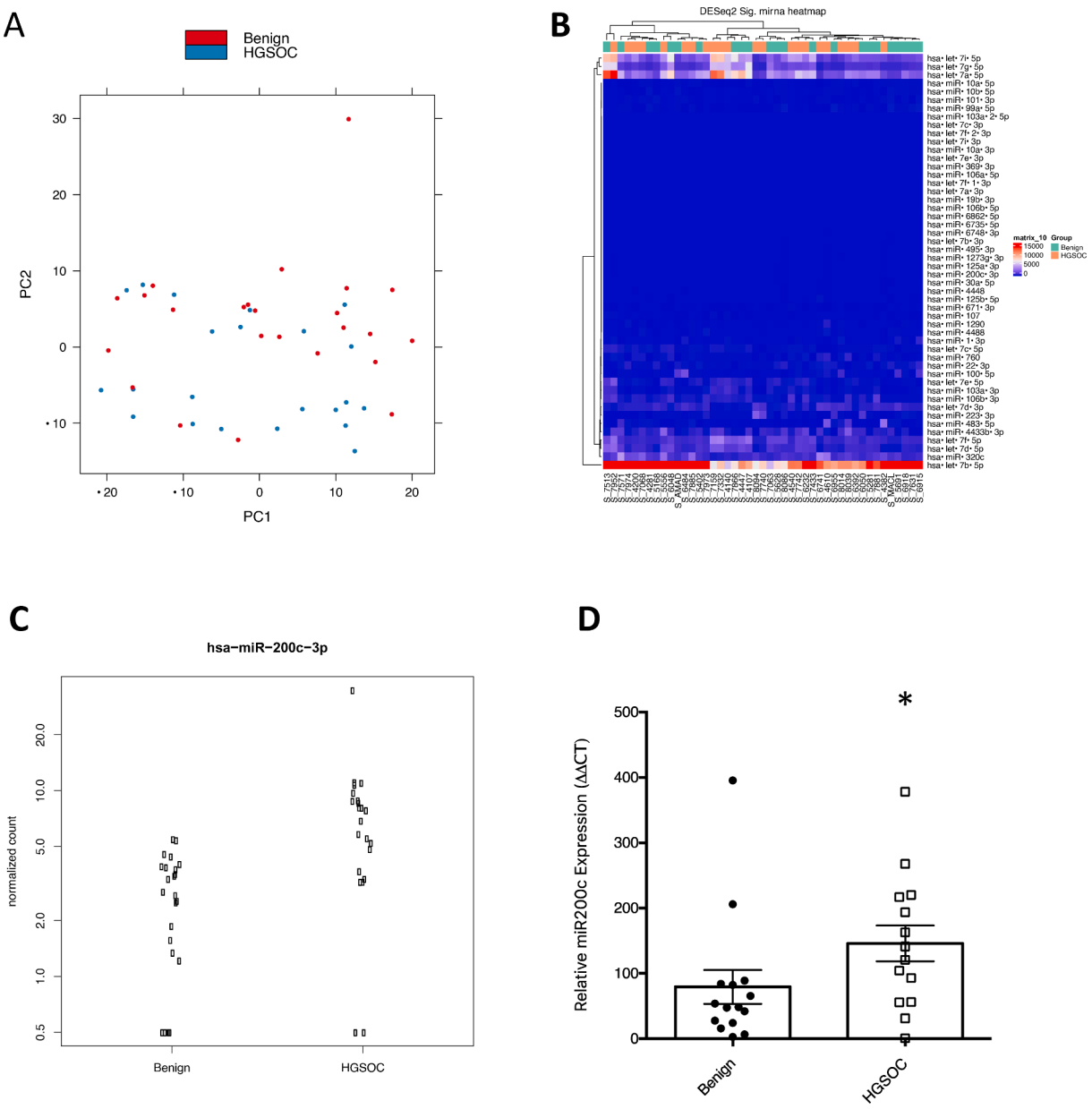

Fig. 1. Targeted next generation sequencing (NGS) for miRNA in plasma from women with high-grade serous ovarian cancer (HGSC). Shows the principle components analysis (PCA) scatterplot of the first two principal components, demonstrating a lack in correlation between the benign controls and cases (HGSC) (A) and a heat map demonstrating unsupervised hierarchical clustering (B) of miRNA expression profiles identified by NGS (DESeq2 analysis) in women with HGSC revealed very weak correlations between cases and controls. As shown in the plot count (C) the miRNA miR200c was found to be increased in plasma from women with HGSC (n = 24) compared to controls with benign ovarian masses (n = 24) (C) in the discovery cohort. Analysis of miR200c expression in plasma from women with HGSC (n = 14) compared to benign controls (n = 16) using quantitative PCR demonstrated a non-significant trend towards increased levels with HGSC (p = 0.06). Quantitative PCR Data is expressed as mean +/− SEM. Mann-Whitney test was used for statistical comparison to controls.

chemotherapeutics (Zuberi et al., 2015; Kim et al., 2007).

In the current study, we used an unbiased next generation screening approach to explore whether targeted next generation miRNA sequencing could detect changes in circulating miRNAs in plasma collected from women with HGSC. Consistent with our previous findings (Pendlebury et al., 2017), and others (Zuberi et al., 2015; Kim et al., 2007) we identified significantly increased expression of the miR200c in the plasma from women with HGSC using RNA-sequencing. In an independent validation cohort we observed increased levels of plasma miR200c by qRT-PCR. Taken together, these two independent studies suggest that circulating levels of the miR200 family in plasma may be differentially expressed in epithelial tubo-ovarian cancer. It is possible that quantitative levels may yield advancing clinical information, such as facilitate cancer staging or provide information about prognosis.

Circulating miRNAs can be detected in whole blood, plasma or serum. This is the first study to describe the altered expression of miR200c in HGSC in miRNA extracted from stored, biobanked plasma. Focused pathway analysis revealed associations between miR200c-3p and six genes involved in cancer.

A strength of this study is the inclusion of only HGSC patients in contrast to previous studies that have analysed miRNAs from patients with a variety of histological subtypes. While these results are promising, important limitations need to be recognized. The extraction of miRNAs from stored plasma samples has raises some technical considerations. It may impact on the quality and precise quantity of RNA extracted from plasma (Poel et al., 2018; Ramón-Núñez et al., 2017).

Table 3
Ingenuity Pathway Analysis of has-miR-200c-3p relationships in ovarian cancer pathways.

Gene symbol/ ID	Confidence	Symbol	Molecule Type
hsa-miR-200c-3p	Moderate (predicted)	EDNRA	transmembrane receptor
hsa-miR-200c-3p	Moderate (predicted)	PIK3CA	kinase
hsa-miR-200c-3p	High (predicted)	PRKACB	kinase
hsa-miR-200c-3p	Moderate (predicted)	PRKAR1A	kinase
hsa-miR-200c-3p	Moderate (predicted)	PRKAR2B	kinase
hsa-miR-200c-3p	Experimentally Observed	PTEN	phosphatase
hsa-miR-200c-3p	Moderate (predicted)	RPS6KB1	kinase
hsa-miR-200c-3p	Moderate (predicted)	VEGFA	growth factor
hsa-miR-200c-3p	High (predicted)	WNT16	other

Particularly around whether to add carrier RNAs, to use equal plasma/serum volumes, to trust 260/280 spectrometry ratios or which reference gene to use is still a point of contention. Thus, a limitation of using plasma RNA is the difficulty to gain accurate, accepted quality control measures. In our study we decided to use equal starting volumes of plasma for the extraction of miRNAs. Additionally, we selected the miRNA hsa-miR-191-5p as our reference gene, following careful assessment of expression of qPCR Cq values. Within our sample set hsa-miR191-5p demonstrated stable expression and performed well as a reference housekeeper, however others have described miR191 as less stable in their assessment (Rice et al., 2015). The relatively small sample size limits the ability to determine the accuracy of miR200c to diagnose HGSC. An additional limitation of the current study is the lack of an asymptomatic, healthy control group and patients with early-stage disease to assess miR200c levels in normal ovarian tissue and normal adjacent tissue in HGSC tumor samples. Further studies of larger cohorts are required to assess the diagnostic potential of circulating miR200 in ovarian cancer.

In conclusion, expression of circulating plasma miR200c was found to be increased in women with HGSC compared to those with benign ovarian masses. Larger validation studies, comparing healthy, matched controls to adequately staged, early HGSC are required to further investigate miR200c as a novel candidate biomarker for the early detection of ovarian cancer.

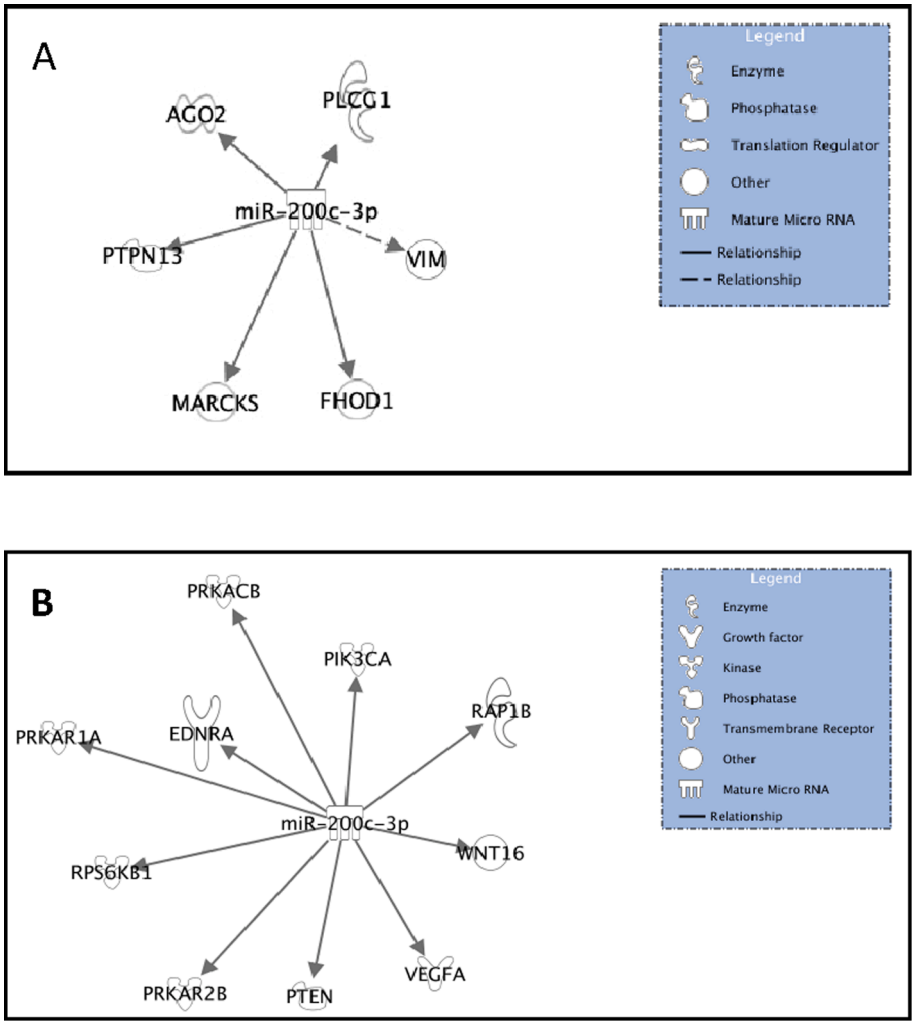

Fig. 2. Ingenuity Pathway Analysis of hsa-miR-200c-3p in the circulation and ovarian cancer. Analysis of pathways associated with miR200c in the plasma and serum (A) and ovarian cancer (B). Six genes were found to be associated with miR-200c-3p (and other miRNAs w/seed AAUACUG) in plasma/serum pathway analysis (A). Focused ovarian cancer pathway analysis revealed miR-200c-3p was associated with the regulation of ten genes (predominantly growth factors) (B).

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Availability of data and materials consent for publication

The RNA seq data has not been deposited on a freely downloaded repository given it has sequence information that could identify participants. The authors are happy to be contacted where the data could be made available if confidentiality agreements are signed between Institutions.

Authors' contributions

NJH: Designed the study, supervised laboratory analysis of samples, helped analyse the results, helped obtain funding and wrote the first draft.

PC: Coordinated the multicenter study including preparing ethics submissions, setting up sites, and overseeing recruitment across sites. Assisted in manuscript preparation.

SB: Performed RNA extraction and RT-PCR of the patient cohorts.

SB and BZ: Supported co-ordination of the recruitment of samples, and collection of data, maintained the patient database.

ST and CW: Helped design the study and interpret the results.

LH: Conceived and designed the study, helped obtain funding, provided overall supervision for the study, helped interpret the results and provided critical input for the manuscript.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.gore.2021.100894.

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