miR-34a is an Intracellular and Exosomal Predictive Biomarker for Response to Docetaxel with Clinical Relevance to Prostate Cancer Progression

Claire Corcoran, Sweta Rani, and Lorraine O'Driscoll*

School of Pharmacy and Pharmaceutical Sciences & Trinity Biomedical Sciences Institute, Trinity College Dublin, Dublin, Ireland

BACKGROUND. Docetaxel-resistance limits successful treatment of castration resistant prostate cancer. We previously demonstrated that extracellular vesicles (exosomes) may play a role in regulating docetaxel resistance. Here, we investigated intracellular and extracellular (exosomal) miRNAs related to docetaxel resistance.

METHODS. Following global miRNA profiling of cell line models of docetaxel-resistance and their corresponding exosomes, we investigated the clinical relevance of four selected miRNAs (miR-598, miR-34a, miR-146a, miR-148a) in four publically available clinical cohorts representing both primary and advanced disease in tissue and urine specimens. One of these miRNAs, miR-34a was selected for functional evaluation by miRNA inhibition and over-expression in vitro. We further assessed the panel of miRNAs for their combined clinical relevance as a biomarker signature by examining their common predicted targets.

RESULTS. A strong correlation was found between the detection of miRNAs in exosomes and their corresponding cells of origin. Of the miRNAs chosen for further validation and clinical assessment, decreased miR-34a levels showed substantial clinical relevance and so was chosen for further analysis. Manipulating miR-34a in prostate cancer cells confirms that this miRNA regulates BCL-2 and may, in part, regulate response to docetaxel. When combined, these miRNAs are predicted to regulate a range of common mRNA targets, two of which (e.g., SNCA, SCL7A5) demonstrate a strong relationship with prostate cancer progression and poor prognosis.

CONCLUSIONS. This study supports the extracellular environment as an important source of minimally invasive predictive biomarkers representing their cellular origin. Using miR-34a as example, we showed that biomarkers identified in this manner may also hold functional relevance. *Prostate 74:1320–1334, 2014.* © 2014 The Authors. *The Prostate*, published by Wiley Periodicals, Inc.

KEY WORDS: **docetaxel-resistance; prostate cancer; exosomes; microRNA; biomarkers; miR-34a; BCL-2**

Grant sponsor: Science Foundation Ireland's funding of Molecular Therapeutics for Cancer, Ireland; Grant number: 08/SRC/B1410; Grant sponsor: EU Co-operation in Science and Technology, ME-HaD; Grant number: BM1202; Grant sponsor: Higher Education Authority's Programme for Research in Third Level Institutions Cycle 5; Grant sponsor: Trinity Biomedical Sciences Institute (TBSI).

Disclosure Statement: The authors have nothing to declare.

*Correspondence to: Lorraine O'Driscoll, School of Pharmacy and Pharmaceutical Sciences & Trinity Biomedical Sciences Institute, Trinity College Dublin, Dublin 2, Ireland. E-mail: lodrisc@tcd.ie

Received 25 March 2014; Accepted 5 June 2014
DOI 10.1002/pros.22848
Published online 22 July 2014 in Wiley Online Library (wileyonlinelibrary.com).

INTRODUCTION

Increased release of prostate-specific antigen (PSA) into circulation has been associated with the onset of prostate cancer. For this reason, its detection in serum has become a universal tool in screening for the disease; particularly in combination with digital rectal examination (DRE) [1]. The use of PSA as a prostate cancer biomarker has faced some limitations [2] however, highlighting the need to identify more potential biomarkers. Ideal biomarkers would be those that could be obtained in a minimally invasive manner and which could perhaps support PSA as a diagnostic, ultimately aiding in earlier detection of prostate cancer together with monitoring disease progression and predicting treatment response.

Castration resistant prostate cancer (CRPC) refers to prostate cancer that has progressed despite castrate serum levels of testosterone [3] and is associated with significant morbidity and mortality [4]. Docetaxel is currently the first-line treatment for patients with CRPC offering some improvement in overall survival in comparison to other anti-cancer agents. Unfortunately, many patients either do not respond or initially respond but then relapse. The failure of taxanes to increase survival beyond the median of 2.5 months may be caused, at least in part, by multidrug resistance (MDR) mechanisms protecting cancer cells against cytotoxic drugs. MDR is frequently attributed with the over-expression of one or several membrane transporter proteins that act as drug efflux pumps [5]. We have previously shown that multidrug resistance protein 1 (MDR-1/P-gp) is over expressed in some docetaxel resistant prostate cancer cell lines and their exosomes [6,7], although mechanisms independent of MDR-1/P-gp expression are also responsible for docetaxel resistance [7].

Growing evidence supports the role of microRNAs (miRNAs) as potential biomarkers for cancer. Their post-translational regulation of gene expression has implicated these short non-coding RNAs (approximately 18–25 nucleotides long) in a range of essential biological activities [8]. Reports on the stability of miRNAs in biological fluids have suggested their latent use as minimally invasive biomarkers [8,9]. These miRNAs may be encapsulated into nano-sized vesicles (known as exosomes) and secreted from cells into the circulation [10]. Recently, studies indicate that exosomal miRNA profiles can reflect their cells of origin [11,12]. Furthermore, previous studies by ourselves and others have demonstrated that exosomes can transfer phenotypic traits from their cells of origin onto secondary cells [6,13,14].

Here, we performed global miRNA profiling of acquired resistant cell line models as representatives of the clinical problem of docetaxel resistance in prostate cancer. Our objective was to investigate their intracellular and extracellular (cell-derived exosomes) miRNA profile as a means of identifying potential clinically relevant biomarkers for docetaxel response/resistance in prostate cancer. Our study reports a direct correlation between the detection of miRNAs in the cells and corresponding exosomes of all cell lines assessed. Of the four miRNAs (miR-598, miR-34a, miR-148a, and miR-146a) identified in this manner, miR-34a was found to have substantial clinical relevance and manipulating its expression confirmed its functional relevance. Considering all four microRNAs as a biomarker signature, we found that a vast number of predicted mRNA targets were common to two, three, or all four of these miRNAs and that some of these targets hold strong clinical relevance with prostate cancer progression. Here, we conclude that the extracellular environment is a significant source for minimally invasive predictive biomarkers that can represent their cells of origin and offer an important starting point for biomarker discovery.

METHODS

Cell Lines and Cell Culture

Prostate cancer cell lines, 22Rv1 (ATCC CRL-2505; androgen-sensitive; from a primary human tumor), DU145 (ATCC HTB-81; androgen-insensitive; from a brain metastasis) and PC3 (ATCC CRL-1435; androgen-sensitive; from bone metastasis) were purchased from the American Type Culture Collection (ATCC). All cells were maintained in RPMI medium (Sigma-Aldrich, Arklow, Ireland) supplemented with 10% fetal bovine serum (PAA), 1% L-Glutamine (Sigma-Aldrich) and at 37°C/5% CO_2. Docetaxel-resistant cell line variants, 22Rv1RD, DU145RD, and PC3RD were generated as previously described [6,7]. Age-matched parent cells (22Rv1, DU145, and PC3) were maintained in culture, unexposed to docetaxel, as controls for all experiments.

Exosome Isolation From Conditioned Medium

All cells were grown in RPMI medium supplemented with 5% of exosomes-depleted fetal bovine serum (dFBS) (PAA), 1% L-Glutamine (Sigma-Aldrich) and 1% penicillin/streptomycin (Invitrogen-Biosciences, Dun Laoghaire, Ireland). FBS was depleted of exosomes by ultracentrifugation for 16 hr. Cells were seeded at a density of 1×10^5 cells/75 cm^2 flask (for DU145 and PC3 variants) and 5×10^5cells/75 cm^2 flask (for 22Rv1 variants). After allowing cells to attach

over-night, medium was replaced and the cells were cultured for three (DU145 cell lines) or five (22Rv1, PC3 cell lines) days in the fresh medium; to approximately 80% confluency. Exosomes were subsequently isolated from conditioned medium (CM) using methods that we recently described [6,13]. The resulting isolates were resuspended in approximately 200 μl PBS and stored at −80°C for subsequent quantification (using BioRad protein assay Dye Reagent) and for inclusion in all analysis detailed. The corresponding cells from which the conditioned media for exosomes isolation was used were washed twice with PBS and pelleted for subsequent analysis.

Transmission Electron Microscopy (TEM)

Exosomes isolated from conditioned media were analyzed by electron microscopy as previously described [15]. Briefly, approximately 10 μl of exosomes samples were placed on parafilm, in duplicate. A 300 mesh copper grid was placed on top of the drop and allowed to stand for 45 min. The copper mesh was subsequently washed thrice in fresh phosphate buffer for 5 min each, fixed in 3% glutaraldehyde for 10 min, washed thrice for 5 min each in dH_2O and contrasted in 2% uranyl acetate. Grids were then stored and examined by electron microscopy at 100 kV using a JEOL JEM-2100 electron microscope.

Immunoblotting

Total proteins were extracted using lysis buffer (Invitrogen). Protein quantification of cells and exosomes was performed using BioRad protein assay Dye Reagent (BioRad-Fannin Ltd, Dublin, Ireland). Protein (50 μg for cellular protein samples and 20 μg for exosomes samples) was separated on 7.5% SDS gels for TSG101 and PDC6I/Alix and 12.5% gels for BCL-2. Immunoblotting involved using the following primary antibodies: PDC6I/Alix [16] (Abcam, Cambridge, UK) and TSG101 [17,18] (Abcam), BCL-2 (Calbiochem-Millipore, Cork, Ireland), β-actin (Sigma-Aldrich). Immobilon Western Chemiluminescent HRP substrate (Millipore, Cork, Ireland) and a Bio-Rad ChemiDoc system were used to visualize the protein bands.

RNA Isolation of Cells and Exosomes

Total RNA was isolated from cells and corresponding exosomes of all three docetaxel-resistant cell line variants and respective aged-parent controls using the miRNeasy mini kit (Qiagen Ltd, Manchester, UK) according to manufacturer's instructions but modified to include the optimized Exiqon protocol for RNA isolation using the miRNeasy kit from biological fluids. Specifically this concerns the volume of QIAzol (700 μl for cells; 750 μl for exosomes) and chloroform (140 μl for cell samples; 200 μl for exosome samples) used and the addition of an extra wash step with RPE buffer.

Global miRNA Profiling of Cells and Exosomes

Taqman miRNA low density arrays (TLDA) (Applied Biosystems -Biosciences, Dun Laoghaire, Ireland) were selected as the platform for miRNA profiling. It consists of two arrays: TLDA panel A and panel B for the assessment of a total of 754 miRNA assays. cDNA was prepared from 3 μl RNA (that was diluted to a constant amount for all cell line variants) following TLDA RT protocol. cDNA (2.5 μl) was pre-amplified and then quantified using Applied Biosystems ViiA7 Real-Time PCR system. Global miRNA profiling data was normalized to the mean of three miRNAs (miR-618, miR-659, and miR-454) that were found not to be significantly altered between the resistant cell line variants and their respective parent controls for both the cells and exosomes and so deemed suitable as an endogenous control.

Validation of Selected miRNAs by qPCR

Following global profiling, miRNAs selected for further validation were based on the following criteria: miRNAs significantly ($P < 0.05$) altered by ≥1.5-fold in the cells and exosomes of at least two docetaxel-resistant cell line variants compared to their aged-matched drug-sensitive parent cell lines. cDNA was prepared from 10 ng cell-derived and exosome-derived total RNA, as we described previously [19]. miR-598 (Cat #4427975, ID: 001988, Applied Biosystems), miR-148a (Cat #4427975, ID: 000470, Applied Biosystems), miR-34a (Cat #4427975, ID: 000426, Applied Biosystems), and miR-146a (Cat #4427975, ID: 000468, Applied Biosystems) were quantified using the cycle threshold (C_T) adjusting to the levels of miR-618 (Cat #4427975, ID: 001593, Applied Biosystems) which showed no significant changes among cells and exosomes of parent compared to the resistant cell line variants and so deemed a suitable for data normalization.

Assessment of miRNA Expression in Clinical Specimens From Publically Available Datasets

As a means of selecting the most appropriate miRNA to further evaluate for functional relevance,

we assessed the clinical impact, of the four validated miRNAs, using publically available datasets on the gene expression omnibus. Of the datasets available, the expression of these miRNAs were initially assessed in clinically localized prostate cancer tissue (n = 21) versus matched benign tissue (n = 21) (GSE36802). To get an indication of the relevance of these miRNAs in the extracellular setting, their expression was assessed in urine samples from prostate cancer patients (n = 9) versus patients with benign prostatic hyperplasia (BPH) (n = 8) (GSE39314). To identify any association between these miRNAs and more aggressive disease, where information was available, patients with known biochemical recurrence (BCR) following radical prostatectomy (n = 30) was compared to those with non-recurrence (n = 53) (GSE26247). Furthermore, these four miRNAs were assessed in a cohort of patients with metastases (n = 14) compared to either primary disease (n = 99) or normal adjacent tissue (n = 28) (GSE21036). An online software tool, MIRUMIR [20], was used to predict the association between miR-34a and overall survival from prostate cancer on the GSE21036 dataset.

miRNA Inhibition/mimic Manipulation in Cells

Docetaxel sensitive (PC3 and 22Rv1) cells were transfected with miR-34a inhibitor (Cat #4464084, ID: MH11030, Applied Biosystems) or miRNA inhibitor negative control (Cat #4464076). These were used at a final concentration of 30 nM and transfected using lipofectamine 2000 (Invitrogen). Similarly, docetaxel resistant (PC3RD and 22Rv1RD) cells were transfected with miR-34a mimic (Cat #4464066, ID MC11030, Applied Biosystems) or miRNA mimic negative control (Cat #4464058). For assessment of protein changes, pellets of transfected cells were collected after 48 hr for subsequent immunoblotting.

Assessing Effects of miR-34a–Regulated Cellular Response to Docetaxel

Following transfection with miR-34a inhibitor, miR-34a mimic or their relevant negative controls for 6 hr, cells were exposed to their approximate IC_{50} concentrations of docetaxel as we previously determined [6]. Following 48 hr incubation with docetaxel, cell viability was assessed using acid phosphate analysis [6].

miRNA Target Prediction and Validation in Publically Available Datasets

The online prediction tool, miRWalk (http://www.umm.uni-heidelberg.de/apps/zmf/mirwalk/index.html) [21] was used to identify predicted mRNA targets of selected microRNAs. Targets identified in a minimum of five prediction programs on miRWalk for each microRNA (miR-598, miR-34a, miR-146a, and miR-148a) were then compared using Venn diagrams (VENNY: An interactive tool for comparing lists with Venn Diagrams. http://bioinfogp.cnb.csic.es/tools/venny/index.html) to identify overlapping mRNA targets in at least two, three, or all four miRNAs. Complete lists of common predicted targets were assessed for clinical relevance in whole blood specimens from prostate cancer patients with advanced castration resistant disease (n = 63) compared to those with good prognosis (n = 31) in the publically available dataset (GSE37199). Targets demonstrating significant association with advanced CRPC in whole blood specimens were then further evaluated in another dataset (GSE16560) and investigated for their association with patients' survival using SurvExpres (http://bioinformatica.mty.itesm.mx:8080/Biomatec/SurvivaX.jsp).

Statistical Analysis

Statistical analysis was performed on Excel. *P*-values were generated using Student's *t*-tests, with $P < 0.05$ considered as statistically significant. Results are displayed as $n = 3 \pm SEM$. GraphPad was used for graph generation. Linear regression analysis and the calculation of R^2 was performed on GraphPad and *P*-values were calculated based on deviation from zero.

RESULTS

Characterization of Isolated Extracellular Vesicles

Extracellular vesicles, isolated from the conditioned medium of all cell line variants, were assessed by transmission electron microscopy to identify the presence of vesicles of approximately 100 nm in diameter indicative of exosomes (Fig. 1A). TSG101 and PDC6I/Alix proteins, considered to be important markers of successful isolation of exosomes [22,23], were detected by immunoblotting of isolates from the conditioned medium of all cell line variants (Fig. 1B).

miRNA Profiling of Docetaxel Resistant Cells and Corresponding Exosomes

Global miRNA profiling of cells and exosomes of the all docetaxel-resistant cell line variants (PC3RD, DU145RD, and 22Rv1RD) and their respective age-matched parent controls (PC3, DU145, and 22Rv1) was performed for 754 miRNAs. All sets of samples were run in biological triplicate. Taking a cut-off point

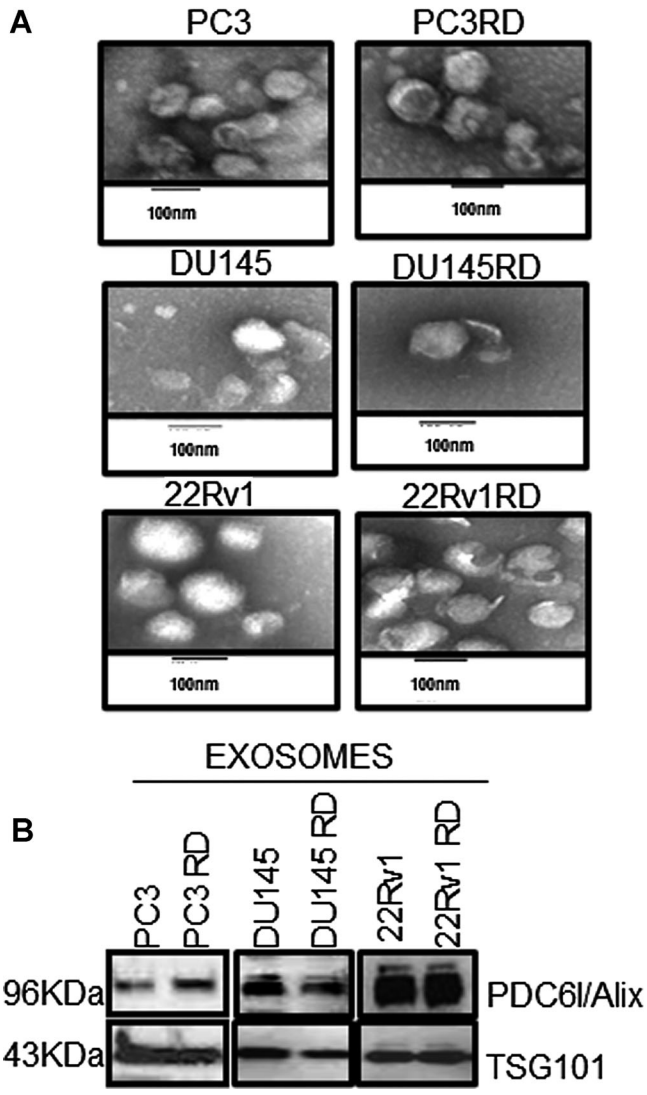

Fig. 1. Exosome confirmation from conditioned media isolates. (**A**) Transmission electron microscopy was performed to investigate size and structure of exosomes; (**B**) Western blotting was performed to assess the expression of common exosomes markers (TSGI0I and PDC6I/Alix) in isolates from PC3, DUI45, and 22RvI cell line variants.

of 35-cycle thresholds (C_T), miRNAs detected $<35C_T$ were considered as "present" where as those with values $>35C_T$ were considered as "undetected." The mean distribution of miRNAs detected for all cell line variants are shown in Figure 2A. Setting the total number of miRNAs detected as an arbitrary one hundred per cent, the corresponding percentages of miRNAs detected in cells and exosomes are shown in Table I. The average percentage of miRNAs detected in cells of all cell line variants (with the exception of PC3) was significantly greater ($P < 0.01$) compared to exosomes (Table I). Furthermore, excluding any miRNAs detected as common to both cells and exosomes, the average percentage of miRNAs solely detected in cells was significantly ($P < 0.01$) greater than that of exosomes only (Table I). Interestingly, there was no significant difference in the percentages of miRNAs commonly detected in both cells and exosomes, averaging to approximately 76.5% for all cell line variants (Table I). Linear regression analysis on miRNAs detected (i.e., $<35C_T$) in both cells and exosomes indicated a significant ($P < 0.0001$) correlation for all cell line variants (Fig. 2B); PC3 ($R^2 = 0.8316$), PC3RD ($R^2 = 0.8144$), DU145 ($R^2 = 0.8140$), DU145RD ($R^2 = 0.7793$), 22Rv1 ($R^2 = 0.7325$), 22Rv1RD ($R^2 = 0.6897$) (Fig. 2B). Hierarchical clustering using normalized fold changes for all biological replicates (denoted by R1, R2, R3) of the three resistant cell line variants compared to respective parent cell lines (PC3RD, 22Rv1RD, DU145RD) was performed. Clustering of each cell line with its corresponding exosomes was observed for all three resistant variant (PC3RD, 22Rv1RD, DU145RD) (Fig. 2C).

Selection of miRNAs for Validation

Following normalization of the global miRNA profiling data, Venn diagrams were used to identify key miRNAs—within cells and exosomes—that may play an important role in docetaxel resistance. Taking a cut-off point of 1.5-fold up- or down-regulated expression in resistant cell line variants compared to their parent cell lines, the common miRNAs (from the three biological replicates) that were identified in cells and exosomes were selected for further assessment. The aim here was to identify both miRNAs common to any given cell line variant and its exosomes, as well as between all cell variants and exosomes. In this way, a total of 12 miRNAs were identified as commonly down-regulated in cells and exosomes in both DU145RD and 22Rv1RD and 44 miRNAs in PC3RD when compared to their respective age-parent control cells and exosomes (Fig. 3A). Overall, one miRNA was found to be down regulated in cells and exosomes of all three cell line variants and 6 other miRNAs were identified as down-regulated in at least two of the three cell line variants compared to age-parent control cells and exosomes (Fig. 3A). The expression of these seven decreased miRNAs in docetaxel-resistant cell line variants compared to their sensitive parent cell lines, as identified from the global profiling, is shown in Figure 3B. Volcano plots demonstrating the spatial expression of all miRNAs assessed is shown in Figure 3C.

While a total of 84, 18, and 5 miRNAs were commonly up-regulated in cells and exosomes of PC3RD, DU145RD, and 22Rv1RD, respectively, there

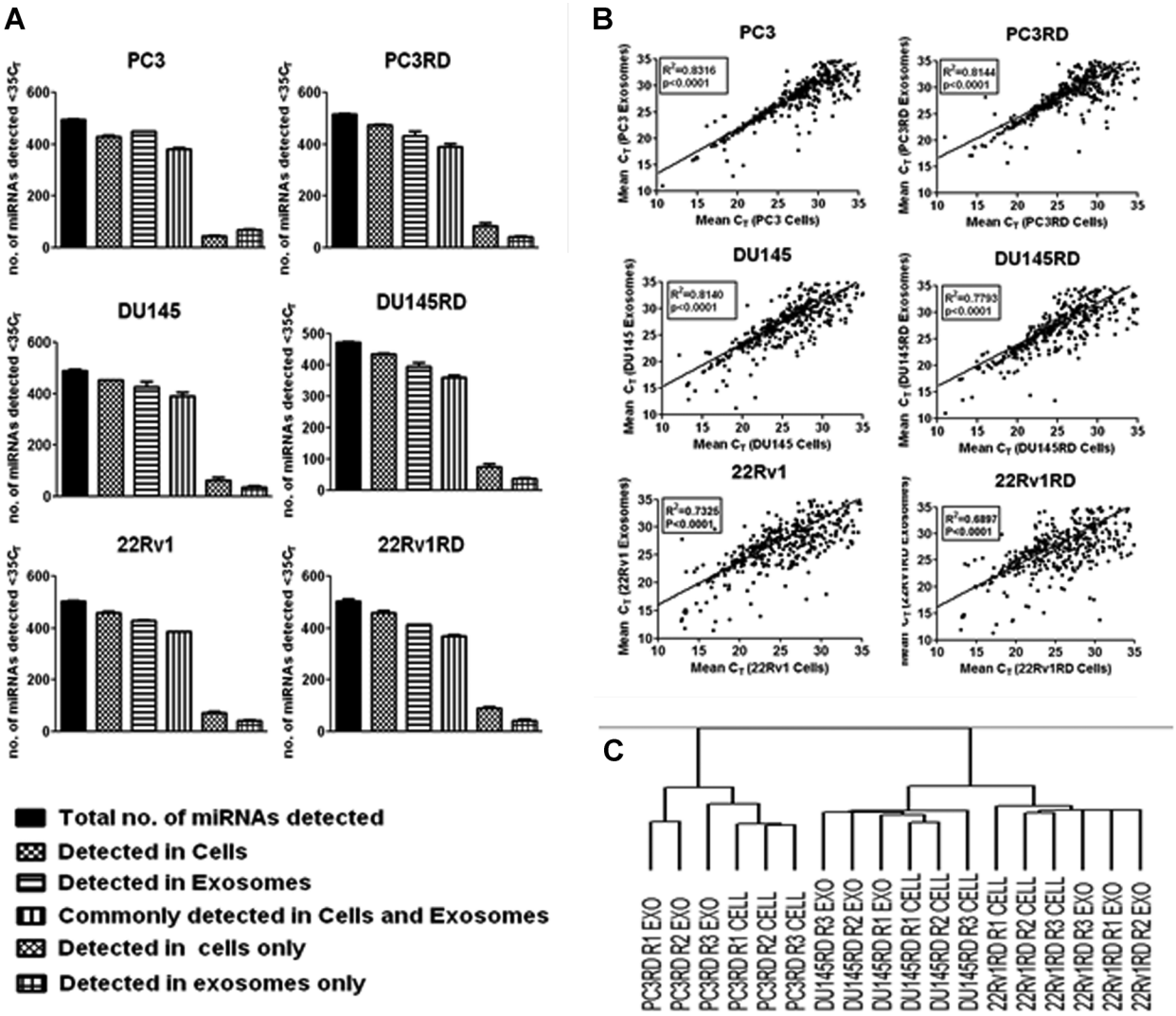

Fig. 2. miRNA profiling of cells and exosomes from all cell line variants. (**A**) The presence of a miRNA was taken at a set cut-off at $35C_T$, thus miRNAs detected below $35C_T$ were considered as "present" where as miRNAs detected beyond $35C_T$ were classified as "undetected." The relevant mean distribution of miRNAs detected in cells and exosomes for each cell line variant is displayed. (**B**) Linear regression analysis was performed to demonstrate the correlation between the mean C_T values of miRNAs detected for cells and corresponding exosomes for each cell line variant. (**C**) Hierarchical clustering of miRNA expression fold changes for docetaxel resistant cell lines (PC3RD, DUI45RD, and 22RvIRD) compared to their respective age-parent controls (RI, R2, and R3 denotes each biological replicate performed).

TABLE I. Percentage and Distribution of microRNAs Detected $<35C_T$ in all Cell Line Variants and Exosomes

Cell line	Detected in cells	Detected in exosomes	Detected in cells and exosomes	Detected in cells only	Detected in exosomes only
PC3	86.2	90.9	77.0	9.1	13.8
PC3RD	91.9	83.9	75.8	16.1	8.1
DU145	91.0	85.4	76.4	14.6	9.0
DU145RD	90.7	85.9	76.6	14.1	9.3
22Rv1	91.4	85.5	76.9	14.5	8.6
22Rv1RD	89.8	86.8	76.6	13.2	10.2
Average	**90.2**	**86.4**	**76.5**	**13.6**	**9.8**

Taking a cut-off point of 35-cycle thresholds (C_T), miRNAs detected $<35C_T$ were considered as "present" where as those with values $>35C_T$ were considered as "undetected." Setting the total number of miRNAs detected as an arbitrary 100%, the corresponding percentages of miRNAs detected in cells and exosomes are shown.

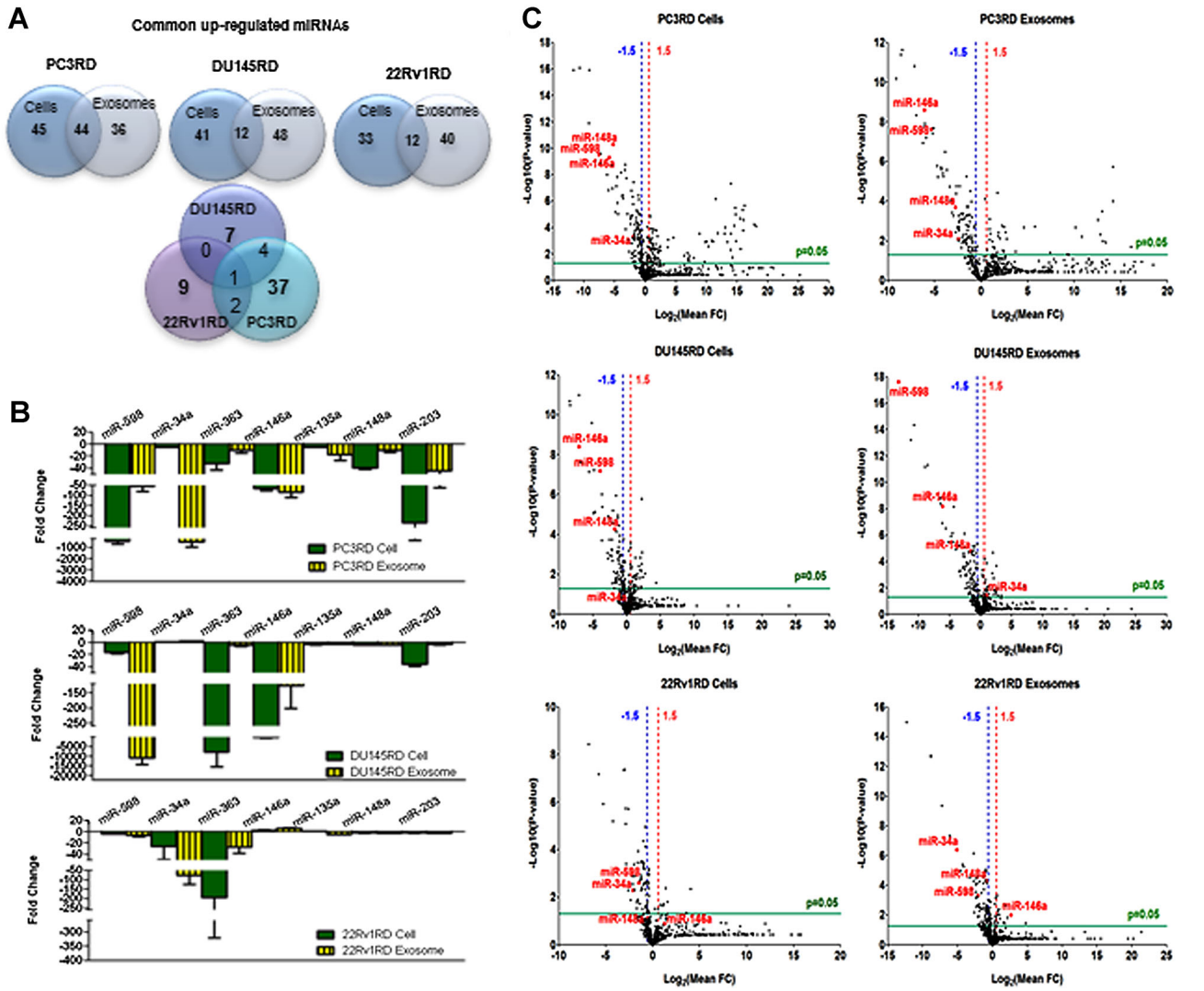

Fig. 3. Assessment of miRNAs fold changes to identify potential miRNAs for further validation. (**A**) Venn diagrams were used to assess the miRNAs that were down-regulated greater than 1.5-fold in the cells and corresponding exosomes of docetaxel resistant cell lines (PC3RD, DU145RD, and 22RvIRD) compared to their respective age-matched parent controls. (**B**) The expression of the seven most substantially down-regulated miRNAs, as identified from the global miRNA profiling, is shown. (**C**) Volcano plots were used to demonstrate the spatial expression of all miRNAs assessed. The x-axis is presented in Log_2 ratio of the fold change of miRNA detection in resistant cell line variants compared to their corresponding sensitive cell lines. The y-axis is the adjusted *P*-value based on $-Log_{10}$. The higher the dot position above the green line (representing $P < 0.05$) the more significant the miRNA fold change. Dots present to the left and right of the blue and red vertical lines are fold changes greater than 1.5. Four miRNAs were selected for independent validation by qPCR as indicated on the volcano plots.

were no miRNAs identified as commonly up-regulated among the three cell line variants compared to their age-parent control cells and exosomes (Fig. S1).

To confirm the results from global miRNA profiling, four miRNAs found to be decreased in the cells and exosomes of at least two of the three docetaxel-resistant cell line variants were selected for validation by qPCR. The expression of miR-598, miR-148a, miR-34a, and miR-146a confirmed the same trends as demonstrated in the global profiling (Table II).

The Clinical Assessment of Selected miRNAs Using Publically Available Data Sets

In order to determine whether any of the four selected miRNAs from the global profiling warranted pursuing for functional assessment, we next assessed if these miRNAs have significant associations in a clinical setting. Using publically available datasets on the gene expression omnibus and analyzing using the GEO2R function, we assessed the expression of

TABLE II. miRNAs Selected for Validation for qPCR

	Cells fold change (mean ± SEM)	*P*-value	Exosomes fold change (mean ± SEM)	*P*-value
miR-598				
22Rv1RD	−2.08 ± 0.21	0.0001	−2.01 ± 0.42	0.0021
DU145RD	−15.65 ± 4.9	0.0301	−15.62 ± 3.14	0.0061
PC3RD	−95.5 ± 19.87	0.0086	−16.93 ± 6.42	0.0492
miR-148a				
22Rv1RD	−1.21 ± 0.5	0.0000	−2.39 ± 0.2	0.0001
DU145RD	−8.32 ± 2.38	0.0172	−3.95 ± 1.06	0.0095
PC3RD	−48.64 ± 3.97	0.0002	−9.93 ± 4.27	0.0626
miR-34a				
22Rv1RD	−7.80 ± 0.61	0.0001	−11.13 ± 0.85	0.0001
DU145RD	−1.08 ± 1.12	0.1378	1.33 ± 0.3	0.3331
PC3RD	−4.68 ± 1.71	0.0294	−5.69 ± 1.98	0.0278
miR-146a				
22Rv1RD	2.14 ± 0.05	0.0000	3.06 ± 0.95	0.0953
DU145RD	−54.42 ± 16.41	0.0279	−81.16 ± 13.42	0.0036
PC3RD	−120.03 ± 97.5	0.2821	−72.65 ± 7.87	0.0007

Validation of miR-598, miR-148a, miR-34a, and miR-146a levels in cells and corresponding exosomes, by qPCR. Fold changes in expression were calculated for all docetaxel resistant cell line variants (22Rv1RD, DU145RD, and PC3RD) compared to age-matched parent controls (22Rv1, DU145, and PC3).

miR-34a, miR-598, miR-148a, and miR-146a in a number of patient cohorts (Fig. 4 and Fig. S2). miR-34a was found to be significantly decreased ($P < 0.05$) in prostate cancer tissue compared to matched benign tissue (Fig. 4A (i)), while a significant increase ($P < 0.01$) of miR-148a was observed (Fig. S2B (i)). No significant difference in miR-598 or miR-146a was observed (Fig. S2A(i) and C(i)). To investigate the potential of our selected miRNAs to be used as circulating and so minimally invasive biomarkers, we examined their expression in urine samples from patients with benign prostatic hyperplasia (BPH) compared with patients with prostate cancer being mindful that the numbers of specimens available was limited. A trend towards ($P = 0.069$) decreased miR-34a levels was observed in urine from prostate cancer patients (Fig. 4A (ii)). miR-148a also showed significantly decreased ($P < 0.05$) levels in urine from prostate cancer patients compared to those with BPH whereas the expression of miR-598 or miR-146a was not significantly altered (Fig. S2A–C (ii)).

In respect to relevance of our selected miRNAs as potential indicators of treatment response/failure, we next assessed their expression in a dataset where information of biochemical recurrence was available. Biochemical recurrence, defined as a rise in serum prostate-specific antigen (PSA) levels following radical prostatectomy and/or radiation therapy, is considered an indicator of more aggressive disease and predictive of early treatment failure [24]. Here, we identified a significant decrease of miR-34a expression ($P < 0.05$) in a cohort of prostate cancer patients experiencing biochemical recurrence compared to patients that had no recurrence (Fig. 4A (iii)). Assessment of miR-598, miR-148a, and miR-146a did not demonstrate any significant discrimination between biochemical recurrence and non-recurrence (Figs. 2A–C (iii)).

As a final assessment of miRNA expression and aggressive disease, we next examined tissue specimens from patients with evidence of metastasis compared to patients with primary localized prostate cancer and also normal tissues. miR-34a was significantly decreased in both primary ($P < 0.01$) and metastatic ($P < 0.01$) disease compared to non-malignant tissue (Fig. 4A (iv)). Furthermore, there was a significant ($P < 0.05$) further decrease in miR-34a expression in metastatic compared to primary disease. Assessment of the other selected miRNAs (miR-598, miR-148a, and miR-146a) displayed some significant alterations in expression, although no consistent trend was observed (Figs. S2A–C (iv)). Using an online tool (MIRUMIR) to predict miRNA association with cancer survival, we identified that low expression of miR-34a, while not statistically significant ($P = 0.075$), tended towards an association with poor survival in prostate cancer (Fig. 4B).

Confirmation of the Functional Relevance of miR-34a

Since, miR-34a demonstrated the most consistent clinical relevance in the four cohorts of patient

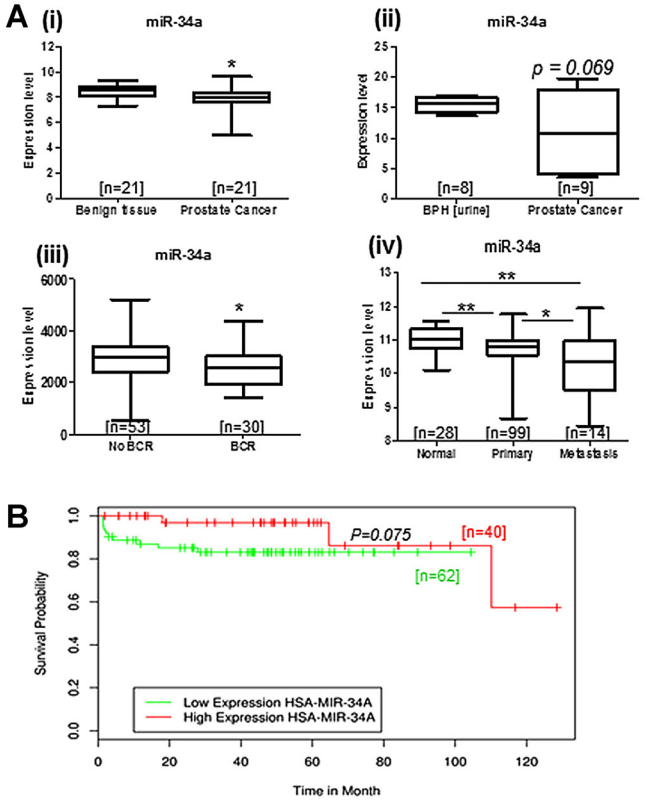

Fig. 4. Clinical assessment of miR-34a using publically available datasets. Using publically available datasets on the gene expression omnibus and analyzing using the GEO2R function, we assessed the expression of miR-34a in a number of patient cohorts. (**A**) (i) miR-34a was found to be significantly decreased ($P < 0.05$) in prostate cancer tissue compared to matched benign prostate tissue. (ii) A trend towards decreased miR-34a levels was observed in urine from prostate cancer patients compared to patients with benign prostatic hyperplasia (BPH). (iii) Here we identified a significant decrease of miR-34a expression ($P < 0.05$) in a cohort of prostate cancer patients experiencing biochemical recurrence (BCR) compared to patients that had no recurrence (no BCR). (iv) miR-34a was significantly decreased in both primary ($P < 0.01$) and metastatic ($P < 0.01$) disease compared to non-malignant tissue. Furthermore, there was a significant ($P < 0.05$) further decrease in miR-34a in metastatic compared to primary disease. (**B**) Using an online tool (MIRUMIR) to predict miRNA association with cancer survival, we identified that low expression of miR-34a, while not reaching statistical significance ($P = 0.075$), tended towards an association with poor survival in prostate cancer. $^{*}P < 0.05$, $^{**}P < 0.01$.

specimens assessed, our final analysis was to further assess the function of miR-34a in our prostate cancer cell lines. Initially, using target prediction software and followed by a literature survey, we focused on B-cell Lymphoma 2 (BCL-2) as a potential target of miR-34a. Assessment of basal BCL-2 expression did not show a significant difference in PC3RD compared to its age-matched parent cells; however, a significant increase in BCL-2 expression was observed in 22Rv1RD compared to 22Rv1 ($P < 0.01$) (Fig. 5A (i)). Inhibition of miR-34a in both parent cell lines (PC3 and 22Rv1) resulted in a significant ($P < 0.01$, $P < 0.05$) increase in BCL-2 protein expression compared to BCL-2 levels in negative control (NC) inhibitor transfected cells (Fig. 5A (ii)). Conversely, mimicked expression of miR-34a in docetaxel resistant PC3RD and 22Rv1RD cells caused a significant reduction in BCL-2 expression ($P < 0.001$, $P < 0.01$) compared to negative control mimic transfected cells (Fig. 5A (iii)). As the innate expression of BCL-2 was significantly increased in 22Rv1RD cells compared to 22Rv1 (where as no significant difference was observed in PC3 compared to PC3RD) we elected to use these cell line variants to assess the effect of miR-34a on both proliferation and response to docetaxel (Fig. 5B). In the presence of miR-34a inhibitor compared to NC inhibitor, no significant differences in the proliferation or response of 22Rv1 to docetaxel were observed (Fig. 5B (i)). Interestingly, while again no significant difference was observed on the proliferation of 22Rv1RD, a significant ($P < 0.01$) decrease in resistance to docetaxel in the presence of miR-34a mimic compared to NC mimic was evident (Fig. 5B (ii)).

Investigating the Prognostic and Predictive Power of Multiple Targeting of miRNAs

While miR-34a was chosen for further functional evaluation of its therapeutic potential as a single biomarker, we next considered whether combinations of miRNAs from our selected panel would hold substantial power as a diagnostic/prognostic and/or predictive signature in prostate cancer. Predicted mRNA targets were identified from a minimum of five programs on MiRWalk. These were subsequently compared using Venn diagrams (Fig. 6A). The complete list of mRNA targets identified common in at least two, three or all four of the miRNAs (miR-598, miR-34a, miR-146a, miR-148a) are shown in Table SI. These common targets were assessed for their clinical relevance in a publically available dataset (GSE31799) comparing the gene expression profile in whole blood from prostate cancer patients with advanced disease ($n = 63$) compared to those with good prognosis ($n = 31$). The most significantly changed mRNAs identified (adjusted $P < 0.05$) are listed in Table SII. Two of these mRNAs, SNCA (alpha-synuclein) and SLC7A5 (solute carrier family 7-amino acid transporter light chain, L system, member 5), are illustrated in Figure 6B as being significantly increased in blood specimens from patients with advanced castration resistant disease. These targets further revealed a significant association with poor prognosis for prostate

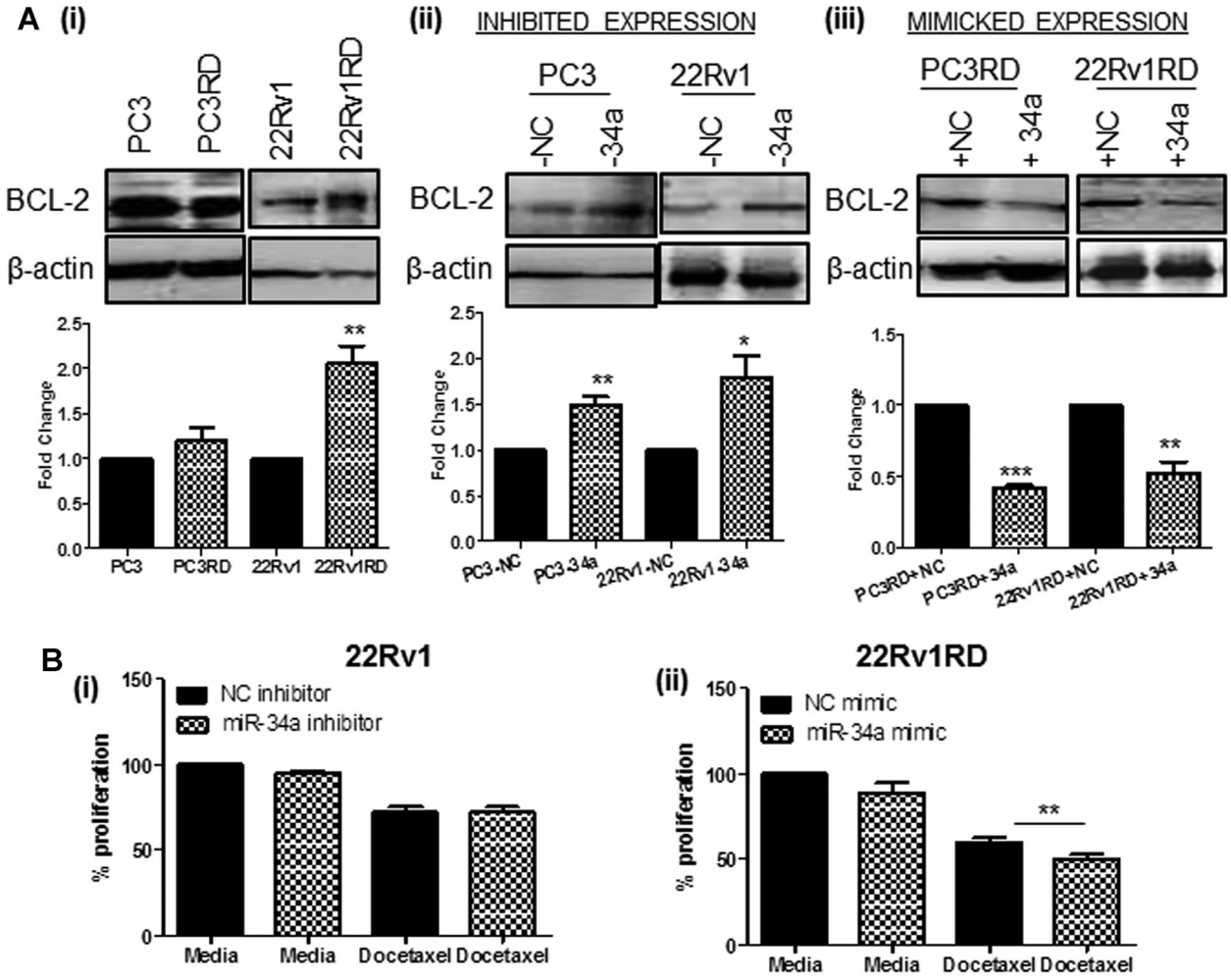

Fig. 5. Mechanism of Action of miR-34a. (**A**) (i) Initial assessment of basal BCL-2 expression did not show a significant difference in PC3RD compared to PC3; however, a significant increase ($P < 0.01$) in BCL-2 expression was observed in 22RvIRD compared to 22RvI. (ii) Inhibition of miR-34a in parent cells (PC3 and 22RvI) resulted in a significant ($P < 0.01$, $P < 0.05$) increase in BCL-2 protein expression compared to BCL-2 levels in negative control (NC) inhibitor transfected cells. (iii) Conversely, mimicked expression of miR-34a in PC3RD and 22RvIRD cells caused a significant reduction in BCL-2 expression ($P < 0.001$, $P < 0.01$) compared to NC mimic transfected cells. (**B**) (i) No significant difference was observed on the proliferation or response of 22RvI to docetaxel in the presence of miR-34a inhibitor compared to NC inhibitor. (ii) While no significant difference was observed on the proliferation of 22RvIRD, there was however a significant ($P < 0.01$) decrease in resistance to docetaxel in the presence of miR-34a mimic compared to NC mimic. n = 3 ± SEM $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

cancer patients in the dataset GSE16505, as shown by Kaplan–Meier survival curves (Fig. 6C).

DISCUSSION

While the first line treatment for castration resistant prostate cancer (CRPC), docetaxel, has often demonstrated initial success in improving overall survival; innate and acquired resistance among patients is continuing to be an immense problem in attempts to circumvent the disease. Substantial findings to date have implicated aberrant miRNA expression in cancer initiation and progression [25,26] and, more recently, the relevance of miRNAs regulating drug resistance has also been reported [27]. Thus, the investigation of miRNAs for use as diagnostic, prognostic and predictive biomarkers for treatment response is now warranted to advance this field. The detection of these miRNAs in an extracellular environment offers the prospect of a minimally invasive and easily attainable biomarker for the clinic. Expanding research in the quest to identify circulating (extracellular) biomarkers has indicated that molecules such as miRNAs may be actively secreted within exosomes and microvesicles with the potential of being taken up into secondary cells [28–30]. We have previously reported that exosomes derived from the conditioned media of docetaxel resistant cell lines can be up taken into secondary

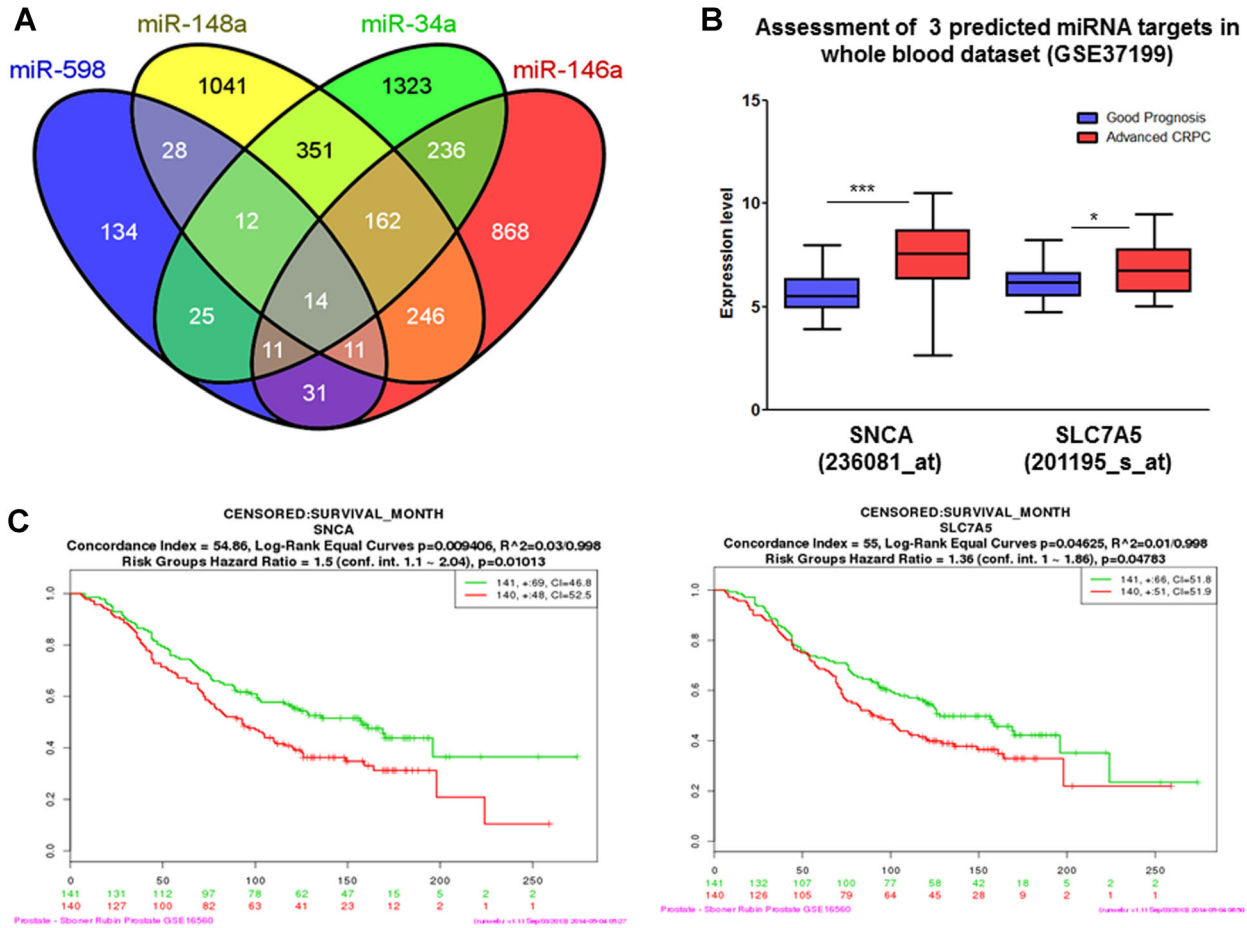

Fig. 6. Combined mRNA targets for miR-598, miR-34a, miR-146a, and miR-148a. (**A**) Venn diagrams were used to assess the common predicted targets for miR-598, miR-34a, miR-146a, and miR-148a. (**B**) An example of two predicted mRNA targets: SNCA (predicted target of miR-598, miR-34a, and miR-148a) and SLC7A5 (predicted target of miR-598 and miR-148a) that held significant association with whole blood from patients with CRPC compared to those with good prognosis (GSE37199). (**C**) SNCA and SLC7A5 also demonstrated significant association with survival of prostate cancer patients in the publically available dataset GSE16560. $^{*}P < 0.05$, $^{***}P < 0.001$.

cells and induce a docetaxel resistance phenotype, at least in part by the apparent transfer of proteins such as MDR-1/P-gp [6]. To further explore the role of exosomes in prostate cancer progression and docetaxel resistance, here we elected to perform global miRNA profiling of the cells and corresponding exosomes from our panel of cell lines with acquired resistance to docetaxel and their age-matched docetaxel sensitive parent cells. Before miRNA profiling was performed, the extracellular vesicles isolated from the conditioned media of all cell line variants were assessed by TEM for size and shape and for the expression of exosomal proteins. The general size of the isolated vesicles for all cell line variants was of approximately 100 nm and taken together with the expression of exosome markers TSG101 and PDC6I/Alix we confirmed the presence of exosomes.

Initial assessment of our miRNA profiling data identified that, of the total miRNAs detected in cells and exosomes, approximately 75% were commonly detected in both cells and exosomes of all cell line variants. Linear regression analysis indicated a positive correlation between the miRNAs detected in all cell lines and their corresponding exosomes. Furthermore, hierarchical clustering using relative fold changes (i.e., the fold change of miRNA expression of each docetaxel-resistant cell line variant compared to its respective age-matched parent control) for all three docetaxel-resistant cell lines clustered together with their corresponding exosomes. This observation is in keeping with that of researchers studying other cancer types [11,12,28]. For example, in a panel of eight miRNAs assessed, Taylor and Gercel-Taylor observed a correlation between miRNA expression in tumors

from patients with ovarian cancer compared to the miRNA expression derived from the serum of the same patients [28]. Furthermore, a strong correlation between the expression of 12 miRNAs in non-small cell lung cancer tumors and the levels of peripheral blood–derived exosomal miRNAs has also been observed [11]. More recently, Xiao et al. [12] performed global mRNA and miRNA profiling on melanoma cells (A375) and human epidermal melanocyte cells (HEMa-LP) and their corresponding exosomes; demonstrating a strong correlation between RNA in each cell line and its exosomes [12]. Taken together our data suggests that exosomal miRNA expression in this prostate cancer setting does, in fact, strongly reflect that of their cells of origin; similar to that reported in other cancer types. This supports the potential use of exosomes derived from biological fluids as a source of biomarkers that may be easily attained with minimal invasion and yet likely to be representative of the clinical situation. Some studies have suggested that exosomal miRNAs are selectively secreted or retained by cells and that their miRNA expression does not reflect the cells of origin [31,32]; while this is not the primary observation of this study, we cannot solely exclude the fact that some miRNAs may be detected at higher levels in the cells compared to exosomes and vice versa. The objective of this study, however, was to focus on miRNAs that had similar intracellular and extracellular profiles with the development of docetaxel resistance as a means of identifying potential biomarkers representative of the cellular phenotype.

As detailed above, we selected four miRNAs for further evaluation based on our profiling data. Specifically, we selected miR-598 for validation by qPCR and assessment in clinical data sets as it was down regulated in all three docetaxel resistant cells and exosomes compared to their respective age-matched controls and has never previously been associated with prostate cancer or drug resistance suggesting that this miRNA may be novel to docetaxel resistance in prostate cancer. We also selected miRNAs that had previous associations with either prostate cancer and/or drug resistance. miR-146a and miR-148a, both of which were decreased in the cells and exosomes of at least two of the three cell line variant pairs assessed, have previously been associated with prostate cancer [33–37] and also drug resistance [36,38–40]. Our fourth miRNA selected for validation was miR-34a. miR-34a's association with prostate cancer [41–43] and drug resistance [44–47] has previously been reported and here we found it to be decreased in both cells and exosomes of two (i.e., 22Rv1RD and PC3RD) out of three docetaxel resistant cell lines. Our subsequent data mining from clinical specimens indicated that miR-34a was the most consistently deregulated miRNA in all clinical cohorts assessed. Specifically, miR-34a was significantly decreased in prostate cancer versus normal tissues; in biochemical recurrence versus non-recurrence tissue and in metastatic versus primary disease prostate tissue. Interestingly miR-34a demonstrated a decreased trend in urine from prostate cancer patients compared to those with BPH. This observation suggests the clinical relevance of extracellular miR-34a although, admittedly, future studies using larger cohorts of patients are necessary to confirm this suggestion. None of the other three miRNAs showed the same level of consistency and/or significant trends in all four of the clinical cohorts assessed; therefore, our subsequent studies assessed the functional relevance of miR-34a.

The overall focus of this article, was not to concentrate solely on the function of specific miRNAs, but rather to investigate the importance of the extracellular environment as a source of predictive biomarkers. In this case particularly, we examined the extracellular vesicle (exosomal) fraction as a means of identifying biomarkers representative of their cells of origin and that potentially could be obtained in a minimally invasive manner if to be used in the clinic. Nevertheless, to confirm that miR-34a for example, has potential as an important biomarker, identified both extracellularly as well as intracellularly, we advanced this study to include some basic in vitro functional analyses.

Online target prediction software identified B-Cell Lymphoma 2 (BCL-2) mRNA to be a target of miR-34a. Mining the literature, we established that studies using other cell lines or in other cancer types have also suggested an association between miR-34a with BCL-2 regulation; thus we elected to assess its relevance here [45,47,48]. We confirmed BCL-2 as a target of miR-34a, by manipulating miR-34a expression in our parent and docetaxel resistant cell lines and subsequently assessing BCL-2 levels. Specifically, upon inhibition of miR-34a in sensitive parent cells (PC3 and 22Rv1) we observed an increase in BCL-2 expression, whereas mimicking miR-34a expression in docetaxel-resistant cells (PC3RD and 22Rv1RD) resulted in decreased BCL-2 expression. Several reports have indicated an association between increased BCL-2 expression and drug resistance [48–51]. In fact, miR-34a regulation of BCL-2 has previously been reported to attenuate paclitaxel resistance in acquired paclitaxel resistant prostate cancer cells [47]. More recently, miR-34a has been shown to induce sensitivity to sorafenib in hepatocellular carcinoma cell lines by inhibiting BCL-2 expression [48], while in vitro and in vivo models of multiple myeloma indicate that the use of synthetic miR-34a mimics can down regulate BCL-2 expression [52]. The association between miR-34a

targeting BCL-2 and regulating docetaxel-resistance in breast cancer has also been reported, although the authors in the breast cancer study found that miR-34a was elevated with docetaxel resistance [45]. Although this observation in breast cancer conflicts with the findings of our study, the other majority of studies support miR-34a being decreased with drug resistance and/or to negatively regulate BCL-2 [44,46–48,52–54]. Here, we found that, while inhibition of miR-34a in 22Rv1 cells did not induce docetaxel resistance, introducing miR-34a into docetaxel-resistant 22Rv1RD cells conferred a level of sensitivity and so enhanced the cytotoxic effects of docetaxel.

The final analysis of this study was to consider the four selected miRNAs (miR-598, miR-34a, miR-146a, and miR-148a) as a potential biomarker signature. Interestingly, we identified a vast number of commonly predicted targets for two, three, or all four of the miRNAs assessed. Furthermore, several of these predicted targets demonstrated significant clinical relevance in whole blood specimens of patients with CRPC compared to patients with good prognosis (indicative of a minimally invasive biomarker option). Two of these mRNA targets of significance, SNCA (predicted target of miR-34a, miR-598, and miR-148a) and SLC7A5 (predicted target of miR-598 and miR-148a), were also found to be significantly associated with poor prognosis in prostate cancer patients. The two predicted targets demonstrated as examples in this study have previously been associated with several cancer types and other pathological conditions. This suggests that the exosomal miRNAs predicted to regulate expression of those genes may hold clinical importance. SNCA has been widely associated with Parkinson's disease and its expression in cancer has also been reported [55–58]. Furthermore, SLC7A5 has previously been associated with both prostate cancer progression [59,60] and other cancer types [61–64]. The strong indication from publically available datasets and in literature that the predicted targets identified from this miRNA signature holds substantial relevance in cancer further supports the importance of the panel of miRNAs identified in this study.

In conclusion, we have identified a panel of miRNAs that are commonly down-regulated in both the cells and exosomes of acquired docetaxel-resistant prostate cancer cell lines. To the best of our knowledge this is the first study to perform global miRNA profiling of both cells and corresponding exosomes in the setting of docetaxel-resistance in prostate cancer and to identify a strong correlation between miRNAs detected in the cells and exosomes of all six cell line variants used in this study. Furthermore, the clinical evaluation of our chosen miRNAs supports the relevance of miR-34a in particular with prostate cancer incidence and progression. The observed decreased expression of miR-34a with biochemical recurrence also suggests its relevance as an indicator of potential early treatment failure. The detection and corresponding decrease of miR-34a in urine from prostate cancer patients compared to patients with BPH suggests its potential as a minimally invasive biomarker although larger patient numbers are necessary to confirm this. Our functional analysis indicates that miR-34a may have a role in influencing cell response to docetaxel in prostate cancer cells, at least partly by its regulation of anti-apoptotic BCL-2. Finally, this study also demonstrates that when considered for their combined relevance, the panel of miRNAs identified in this study may regulate many important mRNAs (such as those discussed in this study among others) that hold substantial clinical association with prostate cancer and, indeed, other cancer types. Leveraging from this body of work, more extensive analyses of serum exosomes including—but not limited to—their miR-34a content, in larger cohorts of patients together with those under-going docetaxel treatment are now warranted.

ACKNOWLEDGMENT

We thank Mr. Neal Leddy Chief Technical Officer Specialist for his TEM expertise.

REFERENCES

1. Catalona WJ, Smith DS, Ratliff TL, Dodds KM, Coplen DE, Yuan JJ, Petros JA, Andriole GL. Measurement of prostate-specific antigen in serum as a screening test for prostate cancer. N Engl J Med 1991;324(17):1156–1161.
2. Gjertson CK, Albertsen PC. Use and assessment of PSA in prostate cancer. Med Clin North Am 2011;95(1):191–200.
3. Galsky MD, Vogelzang NJ. Docetaxel-based combination therapy for castration-resistant prostate cancer. Ann Oncol 2010; 21(11):2135–2144.
4. Berthold DR, Sternberg CN, Tannock IF. Management of advanced prostate cancer after first-line chemotherapy. J Clin Oncol 2005;23(32):8247–8252.
5. Munoz M, Henderson M, Haber M, Norris M. Role of the MRP1/ABCC1 multidrug transporter protein in cancer. IUBMB Life 2007;59(12):752–757.
6. Corcoran C, Rani S, O'Brien K, O'Neill A, Prencipe M, Sheikh R, Webb G, McDermott R, Watson W, Crown J, O'Driscoll L. Docetaxel-resistance in prostate cancer: Evaluating associated phenotypic changes and potential for resistance transfer via exosomes. PLoS ONE 2012;7(12):e50999.
7. O'Neill AJ, Prencipe M, Dowling C, Fan Y, Mulrane L, Gallagher WM, O'Connor D, O'Connor R, Devery A, Corcoran C, Rani S, O'Driscoll L, Fitzpatrick JM, Watson RW. Characterisation and manipulation of docetaxel resistant prostate cancer cell lines. Mol Cancer 2011;10:126.
8. Corcoran C, Friel AM, Duffy MJ, Crown J, O'Driscoll L. Intracellular and extracellular microRNAs in breast cancer. Clin Chem 2011;57(1):18–32.

9. Gilad S, Meiri E, Yogev Y, Benjamin S, Lebanony D, Yerushalmi N, Benjamin H, Kushnir M, Cholakh H, Melamed N, Bentwich Z, Hod M, Goren Y, Chajut A. Serum microRNAs are promising novel biomarkers. PLoS ONE 2008;3(9):e3148.
10. Valadi H, Ekström K, Bossios A, Sjöstrand M, Lee JJ, Lötvall JO. Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. Nat Cell Biol 2007;9(6):654–659.
11. Rabinowits G, Ger el-Taylor C, Day J, Taylor D, Kloecker G. Exosomal microRNA: A diagnostic marker for lung cancer. Clin Lung Cancer 2009;10(1):42–46.
12. Xiao D, Ohlendorf J, Chen Y, Taylor DD, Rai SN, Waigel S, Zacharias W, Hao H, McMasters KM. Identifying mRNA, microRNA and protein profiles of melanoma exosomes. PLoS ONE 2012;7(10):e46874.
13. O'Brien K, Rani S, Corcoran C, Wallace R, Hughes L, Friel AM, McDonnell S, Crown J, Radomski MW, O'Driscoll L. Exosomes from triple-negative breast cancer cells can transfer phenotypic traits representing their cells of origin to secondary cells. Eur J Cancer 2013;49(8):1845–1859.
14. McCready J, Sims JD, Chan D, Jay DG. Secretion of extracellular hsp90alpha via exosomes increases cancer cell motility: A role for plasminogen activation. BMC Cancer 2010;10:294.
15. Lässer C, Eldh M, Lötvall J. Isolation and characterization of RNA-containing exosomes. J Vis Exp 2012;(59):e3037.
16. Shtanko O, Watanabe S, Jasenosky LD, Watanabe T, Kawaoka Y. ALIX/AIP1 is required for NP incorporation into Mopeia virus Z-induced virus-like particles. J Virol 2011;85(7):3631–3641.
17. Fernández-Llama P, Khositseth S, Gonzales PA, Star RA, Pisitkun T, Knepper MA. Tamm-Horsfall protein and urinary exosome isolation. Kidney Int 2010;77(8):736–742.
18. Guescini M, Genedani S, Stocchi V, Agnati LF. Astrocytes and Glioblastoma cells release exosomes carrying mtDNA. J Neural Transm 2010;117(1):1–4.
19. Hennessy E, O'Driscoll L. MicroRNA expression analysis: Techniques suitable for studies of intercellular and extracellular microRNAs. Methods Mol Biol 2011;784:99–107.
20. Antonov AV, Knight RA, Melino G, Barlev NA, Tsvetkov PO. MIRUMIR: An online tool to test microRNAs as biomarkers to predict survival in cancer using multiple clinical data sets. Cell Death Differ 2013;20(2):367.
21. Dweep H, Sticht C, Pandey P, Gretz N. miRWalk–database: Prediction of possible miRNA binding sites by "walking" the genes of three genomes. J Biomed Inform 2011;44(5):839–847.
22. Théry C, Ostrowski M, Segura E. Membrane vesicles as conveyors of immune responses. Nat Rev Immunol 2009;9(8): 581–593.
23. Rani S, O'Brien K, Kelleher FC, Corcoran C, Germano S, Radomski MW, Crown J, O'Driscoll L. Isolation of exosomes for subsequent mRNA, MicroRNA, and protein profiling. Methods Mol Biol 2011;784:181–195.
24. Bruce JY, Lang JM, McNeel DG, Liu G. Current controversies in the management of biochemical failure in prostate cancer. Clin Adv Hematol Oncol 2012;10(11):716–722.
25. Lu J, Getz G, Miska EA, Alvarez-Saavedra E, Lamb J, Peck D, Sweet-Cordero A, Ebert BL, Mak RH, Ferrando AA, Downing JR, Jacks T, Horvitz HR, Golub TR. MicroRNA expression profiles classify human cancers. Nature 2005;435(7043):834–838.
26. Calin GA, Croce CM. MicroRNA signatures in human cancers. Nat Rev Cancer 2006;6(11):857–866.
27. Ma J, Dong C, Ji C. MicroRNA and drug resistance. Cancer Gene Ther 2010;17(8):523–531.
28. Taylor D, Gercel-Taylor C. MicroRNA signatures of tumor-derived exosomes as diagnostic biomarkers of ovarian cancer. Gynecol Oncol 2008;110(1):13–21.
29. Skog J, W rdinger T, van Rijn S, Meijer D, Gainche L, Sena-Esteves M, Curry WJ, Carter B, Krichevsky A, Breakefield X. Glioblastoma microvesicles transport RNA and proteins that promote tumour growth and provide diagnostic biomarkers. Nat Cell Biol 2008;10(12):1470–1476.
30. Hunter M, Ismail N, Zhang X, Aguda B, Lee E, Yu L, Xiao T, Schafer J, Lee M, Schmittgen T, Nana-Sinkam S, Jarjoura D, Marsh C. Detection of microRNA expression in human peripheral blood microvesicles. PLoS ONE 2008;3(11):e3694.
31. Pigati L, Yaddanapudi SC, Iyengar R, Kim DJ, Hearn SA, Danforth D, Hastings ML, Duelli DM. Selective release of microRNA species from normal and malignant mammary epithelial cells. PLoS ONE 2010;5(10):e13515.
32. Mittelbrunn M, Gutiérrez-Vázquez C, Villarroya-Beltri C, González S, Sánchez-Cabo F, González M, Bernad A, Sánchez-Madrid F. Unidirectional transfer of microRNA-loaded exosomes from T cells to antigen-presenting cells. Nat Commun 2011;2:282.
33. Lin SL, Chiang A, Chang D, Ying SY. Loss of mir-146a function in hormone-refractory prostate cancer. RNA 2008;14(3):417–424.
34. Xu B, Feng NH, Li PC, Tao J, Wu D, Zhang ZD, Tong N, Wang JF, Song NH, Zhang W, Hua LX, Wu HF. A functional polymorphism in pre-miR-146a gene is associated with prostate cancer risk and mature miR-146a expression in vivo. Prostate 2010;70(5):467–472.
35. Xu B, Wang N, Wang X, Tong N, Shao N, Tao J, Li P, Niu X, Feng N, Zhang L, Hua L, Wang Z, Chen M. MiR-146a suppresses tumor growth and progression by targeting EGFR pathway and in a p-ERK-dependent manner in castration-resistant prostate cancer. Prostate 2012;72(11):1171–1178.
36. Fujita Y, Kojima K, Ohhashi R, Hamada N, Nozawa Y, Kitamoto A, Sato A, Kondo S, Kojima T, Deguchi T, Ito M. MiR-148a attenuates paclitaxel resistance of hormone-refractory, drug-resistant prostate cancer PC3 cells by regulating MSK1 expression. J Biol Chem 2010;285(25):19076–19084.
37. Murata T, Takayama K, Katayama S, Urano T, Horie-Inoue K, Ikeda K, Takahashi S, Kawazu C, Hasegawa A, Ouchi Y, Homma Y, Hayashizaki Y, Inoue S. miR-148a is an androgen-responsive microRNA that promotes LNCaP prostate cell growth by repressing its target CAND1 expression. Prostate Cancer Prostatic Dis 2010;13(4):356–361.
38. Pogribny IP, Filkowski JN, Tryndyak VP, Golubov A, Shpyleva SI, Kovalchuk O. Alterations of microRNAs and their targets are associated with acquired resistance of MCF-7 breast cancer cells to cisplatin. Int J Cancer 2010;127(8):1785–1794.
39. Tomokuni A, Eguchi H, Tomimaru Y, Wada H, Kawamoto K, Kobayashi S, Marubashi S, Tanemura M, Nagano H, Mori M, Doki Y. miR-146a suppresses the sensitivity to interferon-α in hepatocellular carcinoma cells. Biochem Biophys Res Commun 2011;414(4):675–680.
40. Hummel R, Watson DI, Smith C, Kist J, Michael MZ, Haier J, Hussey DJ. Mir-148a improves response to chemotherapy in sensitive and resistant oesophageal adenocarcinoma and squamous cell carcinoma cells. J Gastrointest Surg 2011;15(3): 429–438.

41. Yamamura S, Saini S, Majid S, Hirata H, Ueno K, Deng G, Dahiya R. MicroRNA-34a modulates c-Myc transcriptional complexes to suppress malignancy in human prostate cancer cells. PLoS ONE 2012;7(1):e29722.

42. Kong D, Heath E, Chen W, Cher M, Powell I, Heilbrun L, Li Y, Ali S, Sethi S, Hassan O, Hwang C, Gupta N, Chitale D, Sakr WA, Menon M, Sarkar FH. Epigenetic silencing of miR-34a in human prostate cancer cells and tumor tissue specimens can be reversed by BR-DIM treatment. Am J Transl Res 2012;4(1): 14–23.

43. Liu C, Kelnar K, Liu B, Chen X, Calhoun-Davis T, Li H, Patrawala L, Yan H, Jeter C, Honorio S, Wiggins JF, Bader AG, Fagin R, Brown D, Tang DG. The microRNA miR-34a inhibits prostate cancer stem cells and metastasis by directly repressing CD44. Nat Med 2011;17(2):211–215.

44. Fujita Y, Kojima K, Hamada N, Ohhashi R, Akao Y, Nozawa Y, Deguchi T, Ito M. Effects of miR-34a on cell growth and chemoresistance in prostate cancer PC3 cells. Biochem Biophys Res Commun 2008;377(1):114–119.

45. Kastl L, Brown I, Schofield AC. miRNA-34a is associated with docetaxel resistance in human breast cancer cells. Breast Cancer Res Treat 2012;131(2):445–454.

46. Vinall RL, Ripoll AZ, Wang S, Pan CX, deVere White RW. MiR-34a chemosensitizes bladder cancer cells to cisplatin treatment regardless of p53-Rb pathway status. Int J Cancer 2012;130(11): 2526–2538.

47. Kojima K, Fujita Y, Nozawa Y, Deguchi T, Ito M. MiR-34a attenuates paclitaxel-resistance of hormone-refractory prostate cancer PC3 cells through direct and indirect mechanisms. Prostate 2010;70(14):1501–1512.

48. Yang F, Li QJ, Gong ZB, Zhou L, You N, Wang S, Li XL, Li JJ, An JZ, Wang DS, He Y, Dou KF. MicroRNA-34a targets Bcl-2 and sensitizes human hepatocellular carcinoma cells to sorafenib treatment. Technol Cancer Res Treat 2014;13(1):77–86.

49. Haarman EG, Kaspers GJ, Pieters R, van Zantwijk CH, Broekema GJ, Hählen K, Veerman AJ. BCL-2 expression in childhood leukemia versus spontaneous apoptosis, drug induced apoptosis, and in vitro drug resistance. Adv Exp Med Biol 1999;457: 325–333.

50. Borgovan T, Bellistri JP, Slack KN, Kopelovich L, Desai M, Joe AK. Inhibition of BCL2 expression and activity increases H460 sensitivity to the growth inhibitory effects of polyphenon E. J Exp Ther Oncol 2009;8(2):129–144.

51. Volm M, Mattern J. Increased expression of bcl-2 in drug-resistant squamous-cell lung carcinomas. Int J Oncol 1995;7(6): 1333–1338.

52. Di Martino MT, Leone E, Amodio N, Foresta U, Lionetti M, Pitari MR, Cantafio ME, Gullà A, Conforti F, Morelli E, Tomaino V, Rossi M, Negrini M, Ferrarini M, Caraglia M, Shammas MA, Munshi NC, Anderson KC, Neri A, Tagliaferri P, Tassone P. Synthetic miR-34a mimics as a novel therapeutic agent for multiple myeloma: In vitro and in vivo evidence. Clin Cancer Res 2012;18(22):6260–6270.

53. Nakatani F, Ferracin M, Manara MC, Ventura S, Del Monaco V, Ferrari S, Alberghini M, Grilli A, Knuutila S, Schaefer KL, Mattia G, Negrini M, Picci P, Serra M, Scotlandi K. miR-34a predicts survival of Ewing's sarcoma patients and directly influences cell chemo-sensitivity and malignancy. J Pathol 2012;226(5):796–805.

54. Weeraratne SD, Amani V, Neiss A, Teider N, Scott DK, Pomeroy SL, Cho YJ. miR-34a confers chemosensitivity through modulation of MAGE-A and p53 in medulloblastoma. Neuro Oncol 2011;13(2):165–175.

55. Ye Q, Wang TF, Peng YF, Xie J, Feng B, Qiu MY, Li LH, Lu AG, Liu BY, Zheng MH. Expression of alpha-, beta- and gamma-synuclein in colorectal cancer, and potential clinical significance in progression of the disease. Oncol Rep 2010;23(2):429–436.

56. Matsuo Y, Kamitani T. Parkinson's disease-related protein, alpha-synuclein, in malignant melanoma. PLoS ONE 2010;5(5): e10481.

57. Bruening W, Giasson BI, Klein-Szanto AJ, Lee VM, Trojanowski JQ, Godwin AK. Synucleins are expressed in the majority of breast and ovarian carcinomas and in preneoplastic lesions of the ovary. Cancer 2000;88(9):2154–2163.

58. Fung KM, Rorke LB, Giasson B, Lee VM, Trojanowski JQ. Expression of alpha-, beta-, and gamma-synuclein in glial tumors and medulloblastomas. Acta Neuropathol 2003;106(2): 167–175.

59. Wang Q, Bailey CG, Ng C, Tiffen J, Thoeng A, Minhas V, Lehman ML, Hendy SC, Buchanan G, Nelson CC, Rasko JE, Holst J. Androgen receptor and nutrient signaling pathways coordinate the demand for increased amino acid transport during prostate cancer progression. Cancer Res 2011;71(24): 7525–7536.

60. Sakata T, Ferdous G, Tsuruta T, Satoh T, Baba S, Muto T, Ueno A, Kanai Y, Endou H, Okayasu I. L-type amino-acid transporter 1 as a novel biomarker for high-grade malignancy in prostate cancer. Pathol Int 2009;59(1):7–18.

61. Kaira K, Toyoda M, Shino M, Sakakura K, Takahashi K, Tominaga H, Oriuchi N, Kanai Y, Oyama T, Chikamatsu K. Clinicopathological significance of L-type amino acid transporter 1 (LAT1) expression in patients with adenoid cystic carcinoma. Pathol Oncol Res 2013;19(4):649–656.

62. Mihály Z, Kormos M, Lánczky A, Dank M, Budczies J, Szász MA, Gy rffy B. A meta-analysis of gene expression-based biomarkers predicting outcome after tamoxifen treatment in breast cancer. Breast Cancer Res Treat 2013;140(2):219–232.

63. Nobusawa A, Kim M, Kaira K, Miyashita G, Negishi A, Oriuchi N, Higuchi T, Tsushima Y, Kanai Y, Yokoo S, Oyama T. Diagnostic usefulness of ^{18}F-FAMT PET and L-type amino acid transporter 1 (LAT1) expression in oral squamous cell carcinoma. Eur J Nucl Med Mol Imaging 2013;40(11):1692–1700.

64. Yanagisawa N, Ichinoe M, Mikami T, Nakada N, Hana K, Koizumi W, Endou H, Okayasu I. High expression of L-type amino acid transporter 1 (LAT1) predicts poor prognosis in pancreatic ductal adenocarcinomas. J Clin Pathol 2012;65(11): 1019–1023.

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at the publisher's web-site.