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Translational applications of microRNAs in cancer, and therapeutic implications

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ABSTRACT

The search for targeted novel therapies for cancer is ongoing. MicroRNAs (miRNAs) display a number of characteristics making them an attractive and realisable option. In this review, we explore these applications, ranging from diagnostics, prognostics, disease surveillance, to being a primary therapy or a tool to sensitise patients to treatment modalities such as chemotherapy and radiotherapy. We take a particular perspective towards miRNAs and their impact on rare cancers. Advancement in the delivery of miRNAs, from viral vectors and liposomal delivery to nanoparticle based, has led to a number of preclinical and clinical applications for microRNA cancer therapeutics. This is promising, especially in the setting of rare cancers.

1. Introduction

Cancer research, from oncogenesis to new therapies, is constantly evolving. Early detection, targeted effective therapies and cure are aims that continue to drive research. One such emerging field in cancer research is that of microRNAs (miRNAs). These are short, non-coding RNAs of 22 nucleotide length that have extensive influence over cell processes. Dysregulation of miRNA expression in cancers is now being exploited for potential therapeutic applications.

The project RARECARE [1] (Surveillance of Rare Cancers in Europe) defines rare cancers as having an incidence of less than 6/100,000 per year, and comprise 22% of cancer diagnoses annually. However, they bear a disproportionate burden of lower survival. The rare cancers on aggregate have a 5-year survival of 47% compared to 65% in the common cancers. There are generally no public screening programs for rare cancers, and patients often present late with advanced disease. As such, the emerging field of miRNA research may have much to offer rare cancer studies, from expanding their molecular characterisation to offering new therapeutic targets.

From a drug development perspective, agents need to fulfill key characteristics [2], including unmet clinical need, a clear clinical benefit over existing therapies, and documented safety and efficacy profiles. miRNAs, with their small, known sequences, often completely conserved between species, are therefore becoming attractive therapeutic options.

This review will examine the translational applications of miRNAs in cancer, with a perspective towards rare cancers. We will also present work from our laboratory highlighting the potential of miRNA replacement therapy in the *in vivo* setting.

1.1. miRNA biogenesis

miRNAs were initially regarded as non-critical non-coding RNAs and were first characterised in Caenorhabditis elegans, a roundworm [3]. *lin-4*, the first discovered miRNA, was found to encode for short transcripts (21–60 nucleotides long) that bound to the 3′ untranslated region (UTR) of *lin-14* mRNA. This resulted in

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repression of translation and lin-14 protein down regulation, showing that miRNAs function in RNA silencing.

In the decades since their discovery, the regulation of gene expression by miRNAs has been found to be post transcriptional. Varying complementarity with their target transcripts leads to degradation or repression of their target messenger RNAs [4]. This is known as RNA interference (RNAi). miRNAs are encoded by their own genes that reside either in non-coding regions of the genome or in non-coding regions of protein coding genes (introns). In their conventional biogenesis pathway, they are synthesised in the cell nucleus from a promoter in a long precursor form (primary-miRNA) by RNA polymerase II. This form is spliced, capped and polyadenylated to form primitive miRNA (pri-miR). Pri-miR undergoes processing by Drosha (the ribonuclease RNase III enzyme) and the protein Pasha, to form pre-miRNA. These pre-miRNAs are exported into the cell cytoplasm via Exportin-5, and processed by the RNase III enzyme Dicer to form a mature hairpin precursor miRNA duplex. The hairpin precursor is loaded into an Argonaute (AGO) protein of the silencing complex as double stranded RNA, with further maturation expelling the passenger and mostly unused miRNA, leaving a mature RNA silencing complex [5]. Protein synthesis is repressed when AGO is attached to the 3′ UTR of target genes.

2. miRNAs and cancer diagnostics

Aspects of miRNAs, including their presence and stability in a range of biological materials other than the parent tumour, specificity as tissue markers, offer great opportunities for cancer diagnostics.

2.1. Stability of miRNAs

miRNAs have been found to be robust and stable in easily available biological material [6]. Chen et al. [7] exposed human serum to a variety of extreme states, including pH, temperature, and freeze/thaw cycles, and found that miRNAs were still quantifiable via RT-qPCR. Additionally, half of the miRNA RT-qPCR products from a lung cancer cell line remained intact following a 3-h exposure to RNAse A, in contrast to large molecular weight RNAs that were rapidly degraded. The stability of miRNAs was also tested by Mraz et al. [8] in clinical samples of B lymphocytes from chronic lymphocytic leukaemia. Freshly isolated RNA was screened for a panel of 29 miRNAs, and then again 14 days later after storage in −80 °C. RT-qPCR was repeatedly performed on stored RNA samples for 10 months. A high stability of isolated miRNAs and cDNAs was observed.

2.2. Specificity as tissue markers

miRNAs have been found to be tissue specific [9,10] and several studies have suggested that miRNAs may be useful in the diagnosis of cancers of unknown origin. Cancers of unknown primary origin comprise a very aggressive disease with poor prognosis. They have a low incidence, constituting 2–5% of newly diagnosed cancers, with a mean survival of less than a year. Additionally, only 30% of patients with cancers of unknown origin will have the primary site of origin identified [11]. Lu et al. profiled 217 mammalian miRNAs in 334 diverse samples of normal tissues and cancers, including colon, liver and pancreas [12]. It was observed that there was a general down-regulation of miRNAs in tumours compared with normal tissues. The authors postulated that global under-expression of miRNAs in cancers may reflect a state of decreased cellular differentiation. They developed a miRNA profiling reference for each cancer studied. Seventeen poorly differentiated cancers, where the diagnosis could not be made through histological examination alone were tested using the established miRNA references. Twelve of the seventeen poorly differentiated cancers were correctly identified by this method. Rosenfeld et al. performed a similar study using a profile of 48 miRNA markers and demonstrated an accuracy of 90% among 22 tissue specimens, including 130 metastases [13]. Using miRNAs in a diagnostic manner has reached a commercial setting, with Rosetta Genomics, a molecular diagnostics company, devising a clinical test to measure expression levels of 48 miRNAs by quantitative reverse transcription-PCR (RT-qPCR), to predict tissue of origin. An algorithm was developed where test predictions correctly identified tissue of origin in 85% of cases.

2.3. miRNAs are identifiable in biological materials other than primary cancers

Another way miRNAs may be utilised in a non-invasive manner to diagnose cancer is through their detection and profiling in biological material such as saliva, stool and plasma. Kalimutho et al. [14] identified stably expressed miRNAs in the faecal microenvironment. miR-144 was highly expressed in stool samples and their paired surgical cancer specimens. The sensitivity and specificity of miR-144 as a novel marker of colorectal cancer was 74% and 79% respectively. Another similar study [15], found significantly higher miR-21 and miR-92a levels in stool. The authors had previously identified these miRNAs to be up-regulated in colon cancer tissue specimens compared to surrounding normal tissue. For miR-92a, sensitivity and specificity were 72% and 56% respectively. Particular benefits of stool miRNA detection include their resistance to degradation, and that colonocytes are continually shed into the stool. This is in comparison to examining stool for occult blood, which may be lost intermittently and non-homogenously.

In squamous cell lung cancer, Xing et al. [16] developed a panel of miRNAs biomarkers for detection in sputum. This miRNA panel yielded a sensitivity of 73% and a specificity of 96%. Again, this was with the explicit aim of developing a minimally invasive method of detecting lung cancer. Similar findings were identified for non-small cell lung cancer and lung adenocarcinoma [17–19].

Circulating miRNAs are universally accessible and potentially of great utility. In breast cancer, Roth et al. examined the sera of women with primary and metastatic breast cancer for miRNAs with known association to breast cancer based on breast cancer cell lines and primary tumour tissue [20]. miR-155 levels in serum significantly discriminated women with primary breast cancer from healthy controls and miRs 10b, 34a and 155 significantly separated metastatic breast cancer patients from healthy controls. Zearo et al. [21] recognised that miRNA profiles in serum do not always mirror that found in cancer tissue, and that focusing on tissue specific miRNAs may exclude other important miRNAs. They identified serum miR-484 as being significantly higher in the sera of breast cancer patients compared to healthy controls. However, there was no differential expression of miR-484 in breast cancer tissue specimens compared to normal breast tissue. The authors recognise that miR-484 could be useful as an alternative means to identify patients with early breast cancer.

Circulating miRNAs may be close to being used in a clinical context. For instance, circulating miRNAs in primary breast cancer

patients may predict for onset of metastasis, progression free survival or overall survival [22]. Circulating miRNAs are not limited to being examined only from whole serum. Ashby et al. [23] developed a refined method for separating different miRNA carriers such as proteins, lipoprotein particles, and exosomes in serum. Using sera from healthy controls and cancer patients, selected miRNAs were quantified via RT-qPCR, with distinct differences in the profiles between the control and cancer cases.

2.4. Diagnostic miRNA signatures

Screening for cancer utilising miRNA signatures has reached the pre-clinical and clinical testing stages. One pre-clinical effort is the mir-Test [24]. The idea behind this test was to formulate a sensitive, non-invasive method of detecting early lung cancer in a high risk group (heavy smokers, greater than 50 years of age). This is compared to patients being monitored with low dose chest CT scanning. A non-invasive and reliable test is particularly important in this population, given the size of the at risk population (smokers, over 50), and the costs and feasibility concerns of monitoring this group through CT. This "mir-Test", which comprises of a panel of serum-detected 13 miRNAs associated with lung cancer, was validated by comparing lung cancer patients and healthy subjects. The mir-Test has shown early promise, with all the patients surgically treated for a benign tumour (albeit a low number) having a negative mir-Test, and there is a 70% sensitivity in those with established lung cancer at different stages. Furthermore, in patients with Stage 1 non-small cell lung cancer (NSCLC), there was a statistically significant decrease in their mir-Test at five months post-surgery compared to one month post-surgery. The authors acknowledge the limits of the study, chiefly that it is monocentric, and as such, the study has been expanded into a multi-centre lung cancer screening trial, enrolling 10000 high risk patients over eight centres, where the patients will be screened with both CT and the miR-Test.

One clinical trial underway using circulating miRNAs as a screening tool for lung cancer (Plasma microRNA Profiling as First Line Screening Test for Lung Cancer Detection: a Prospective Study) is ongoing and currently in Phase 3 [25]. This trial is seeking to establish the utility of this test as a screening tool for lung cancers in heavy smoker volunteers through plasma miRNA profiling as a first line, and demonstrate whether there is a reduction in false positives compared to conventional CT screening.

2.5. Rare cancer perspective

Adrenocortical carcinoma (ACC) is a rare disease, with an incidence of 4–12 per million of the population [26]. It presents late and prognosis is poor. Five-year survival over all stages approaches 35%. It is a cancer in which miRNA profiling has improved molecular understanding of the disease, and from which therapeutic targets are emerging.

While ACC is rare, adrenal incidentalomas are not, with an incidence of around 3% in the population above 50 years [26]. The diagnosis is sometimes only made following adrenalectomy. A recently published update for guiding the management of adrenal incidentalomas provides an evidence based framework for the work-up and management of adrenal masses [27]. In this guideline, radiologic and hormonal measures inform the suggested management advice. In ACC, Patel et al. [28] selected a panel of five miRNAs drawn from ACC miRNA profiling studies. Serum was obtained from 22 patients with adrenal adenomas and 17 patients with ACC. All five panel miRNAs were detected, with significantly greater serum levels of miR-34a and miR-483-5p detected in the ACC cohort. Similarly, Szabo et al., demonstrated that five of eight miRNAs reported as differentially expressed in ACC in the literature, were overexpressed in preoperative plasma samples of patients with carcinomas versus adenomas [29]. This shows that, for adrenal masses facing diagnostic uncertainty, miRNA based diagnostics in addition to radiologic and hormonal assessment can be a very valuable tool.

3. miRNAs and cancer prognostics

miRNAs have been used to prognosticate the clinical courses of cancers [30,31]. Calin et al. were amongst the first to correlate miRNA signatures with prognostic features in patients with chronic lymphocytic leukaemia (CLL) [32]. In CLL, low *IgVh* (immunoglobulin variable region heavy chain) gene mutation status and high ZAP-70 (70-kD zeta-associated protein) protein expression correlated with aggressive disease. A miRNA signature (comprising 13 genes) was identified to be associated with these prognostic factors. In hepatocellular carcinoma, a decreased level of miR-26 was an independent predictor of survival, and it was correlated with a shorter survival [33].

As an adjunct to predicting survival, miRNA profiles have also been examined for their roles for patients' heterogeneous responses to chemotherapy and radiotherapy. This is an extension of prognosis in part, identifying subsets of patients that are not likely to have their cancer respond to therapy. In this way, toxic treatment for which there is no likely cancer response might be avoided.

3.1. miRNAs and chemoresistance

Yuan et al. [34], in a study of patients with diffuse B cell lymphoma, identified miR-125b and miR-130a as biomarkers of resistance to R-CHOP (rituximab, cyclophosphamide, doxorubicin, vincristine and prednisolone) therapy. These two miRNAs were shown to be over-expressed in more drug resistant cases. This observation guided the dynamic management and treatment of the study patients. In the patients demonstrating no disease recurrence in the three-year follow up period, serum levels of miR-125b and miR-130a decreased at 2 months following treatment and normalised at one year. In one patient with initial response/remission, these miRNA levels decreased, but increased again at 12 months, whereupon R-CHOP therapy was recommenced. Sera levels of the miRNAs dropped according to response to treatment. In another patient, whose miRNA levels did not drop and had primary refractory disease, the R-CHOP regimen was changed to a second line treatment at 3 months. Though the sample size was small, the authors found that the serum miRNA levels changed ahead of clinical diagnosis of recurrence or progression. Other studies have sought to define miRNA signatures associated with chemoresistance for the purpose of treatment stratification. Villaruz et al. [35] profiled miRNA expression in metastatic melanoma patients receiving carboplatin/paclitaxel. Objective response rates in chemotherapy naïve metastatic melanoma patients were only 18–20%. They identified high miR-659-3p expression as delineating responsive patients compared to patients with stable disease.

Others have utilised chemoresistance associated miRNAs as therapeutic targets. Kovalchuck et al. [36] found that the

doxorubicin resistant breast cancer cell line MCF-7 cells had higher levels of P-glycoprotein than parental MCF-7 cells. Computational analysis of the 3′ UTR of the *MDR1* (multidrug resistance protein 1) gene showed a binding site for miR-451, which was then found to be under-expressed in doxorubicin resistant MCF-7 cells. Following transfection with miR-451 mimics, the doxorubicin resistant MCF-7 cells showed increased sensitivity to doxorubicin, with the IC50 being two and a half times lower than that of negative controls.

3.2. miRNAs and radioresistance

Resistance of cancers to radiotherapy treatment has also been found to be influenced by miRNAs [37,38]. Ma et al. [39], treated four NSCLC cell lines with sequential doses of radiation. They found a 2.5-fold up-regulation of miR-95 in the NSCLC cell line that demonstrated the most radioresistance. Further to this, inhibition of miR-95 expression in the radioresistant cell line improved apoptosis efficiency following repeat radiotherapy. In a mouse model with miR-95 knockout cell xenografts, the miR-95 knockout xenografts exhibited decreased tumour volume compared to the control cell xenografts following irradiation.

3.3. miRNAs as a surveillance tool

An important application for miRNA is its role as a surveillance tool. Sapre et al. [40] applied a panel of miRNAs acting as a signature for urothelial bladder carcinoma, to a cohort of patients comprised of benign controls, patients with active cancer, and patients with prior bladder cancer under active surveillance. They were able to detect bladder cancer with a sensitivity of 88% and specificity of 48%. Additionally, they projected that by using a miRNA screening process, the established and invasive method of cystoscopy for screening bladder cancer patients, would be reduced by 30%.

3.4. Rare cancer perspective

Gallbladder carcinoma (GBC), with an incidence of between 0.8 and 1.2% has a five year survival of 5% [41]. This has been attributed to its vague, innocuous symptomatology, and patients present late with metastatic disease. Kono et al. [42] examined the expression of miR-155 in GBC. They identified high expression of miR-155 being greater than 1.5 fold in GBC, compared to normal gallbladders. Furthermore, there was a median 20-month difference in disease specific survival between patients with a high miR-155 expression level compared to those with a low miR-155 level, as well as longer overall survival. A higher miR-155 level was significantly associated with lymph node metastasis and vessel invasion. GBC cell lines were transfected with miR-155 inhibitors, mimics, or negative controls. Cells treated with miR-155 inhibitors showed significantly decreased proliferation and invasiveness compared to negative controls, and cells treated with miR-155 mimics displayed increased proliferation and invasiveness compared to negative controls. The authors point to the potential of miR-155 in the setting of gallbladder cancer, since it usually presents as advanced disease. Benefits may include informing disease prognosis and miR-155 may also be a potential therapeutic target.

3.5. Table 1: miRNAs associated with prognosis and diagnosis in selected rare cancers

Rare Cancer	microRNAs associated with more aggressive disease	microRNAs associated with diagnosis
Gallbladder cancer	miR-26a* [43] – downregulated miR-145* [44] – downregulated miR-125b* [45] – downregulated miR-155 [42] – upregulated miR-182 [46] – upregulated miR-138* [47] – downregulated miR-146b-5p [48] – downregulated miR-34a [49] – downregulated miR-1, 145 [50] – downregulated miR-20a [51] – upregulated	miRs-21, 187, 202 [52] – upregulated miRs-143, 335, let-7a [52] – downregulated
Mesothelioma	miR-34b/c* [53] – downregulated miR-29c [54] – downregulated miR-31 [55] – upregulated miRs-21-5p, 23a-3p, 30e-5p, 221-3p, 222-3p [56] – upregulated miR-31-5p [56] – downregulated miRs-17-5p, 30c [57] – downregulated	miR-625 [58] – upregulated miRs-200c, 200b, 141, 429 [59] – downregulated miRs-30e-3p, 103a-3p [60] – downregulated miR-21 [61] – upregulated miR-126 [61] – downregulated miR-193 [62] – upregulated
Adrenocorticocarcinoma	miR-205* [63] – downregulated miR-195 [64] – downregulated miR-483-3p [64] – upregulated	miRs-210, 181b, 100 [29] – upregulated miR-675 [65] – downregulated miR-483-5p [66] – upregulated miR-503 [67] – upregulated miRs-34a, 497 [67] – downregulated
Medullary thyroid cancer	miRs-10a, 200b/-200c, 7, 29c [68] – downregulated miRs-130a, 138, 193a-3p, 373, 498 [68] – upregulated miR-21 - upregulated [69] miR-224 [70] – downregulated miRs-183, 375 [71] – upregulated	
Parathyroid carcinoma		miRs-296, 139 [72] – downregulated miR-222 [72] – upregulated miR-126* [73] – downregulated

* Indicates use as a therapeutic in *in vivo* studies.

4. miRNAs as a therapeutic approach

miRNA therapy has been explored in a number of settings though many challenges exist to its utilisation as a therapeutic. These include poor cellular intake, a lack of tumour targeting delivery vehicles, and unwanted side effects. Where pathogenic miRNA overexpression is present, miRNA sponge therapy has been explored. For miRNAs that are underexpressed in a phenotype and restoration of expression is desired, techniques to deliver miRNA mimics include liposomal delivery, viral delivery, and nanoparticle delivery.

4.1. microRNA sponge therapy

miRNA sponge therapy is a potential therapeutic approach when cancers feature overexpressed miRNAs. The aim is to achieve miRNA loss of function through binding to and inactivation of miRNAs. It has been found that naturally occurring non-coding RNAs can function as miRNA sponges in plants, animals and humans [74]. One example of a miRNA sponge effect, is Hepatitis C infection (HCV) [75], where the HCV virus acts as a miRNA sponge, and the disease proliferates. HCV relies on liver specific miR-122 for replication. miR-122 binds to the 5′ UTR of HCV genomic RNA, with viral protein translation stimulated and the HCV genome is shielded from degradation [76]. This entire mechanism is being targeted as a therapy with a miR-122 inhibitor, miravirsen, that is currently in phase II clinical trial in Europe for treatment naïve and non-naïve patients. Luna et al. [76], in examining miR-122 targets following virus infection, found a de-repression of miR-122 targets, and suggest that the HCV RNA acts to sequester miR-122 and play a "sponge-type" role similar to competing endogenous RNAs.

Miravirsen itself is an example of a third generation locked nucleic acid (LNA) antisense oligonucleotide inhibitor. In third generation LNAs, the modifications enable a greater plasma stability, RNA-like structure, and inhibit RNAse H digestion [77]. LNAs have been utilised in many in vitro and *in vivo* studies to model knockdown and replacement miRNA therapy.

Moshiri et al. have modified adeno-associated viral vectors (AAVs) to express multiple binding sites for miR-221 [78]. miR-221 was shown to be upregulated in hepatocellular carcinoma (HCC) and associated with greater pathogenicity in the disease. The constructs enabled down-regulation of miR-221 to endogenous levels with a de-repression of the miR-221 target CDKN1B (Cyclin-dependent kinase inhibitor 1B)/p27 protein.

4.2. Liposomal delivery

Liposomes are spherical lipid vesicles, with a phospholipid bilayer structure. Hydrophobic and hydrophilic drugs can be incorporated into liposomes and they are being developed currently to be pH, temperature and magnetic sensitive. A number of Phase III trials are ongoing involving liposomal drug formulations including drugs such as danorubicin in acute myeloid leukaemia, cisplatin in gastric and pancreatic cancers, and doxorubicin in hepatocellular cancer [79].

One aspect of liposomal delivery lies in the particle's propensity to accumulate in the liver. A mouse study utilising a chromogenic *in situ* hybridisation technique identified levels of miR-34 in tumour, liver and bone marrow increased by greater than two orders of magnitude, 2 minutes after the liposomal therapy carrying miR-34 mimics (MRX34) was delivered by tail vein injection [80]. MRX34 is a liposomal miR-34 mimic that had entered a multicentre Phase I clinical trial. However, the trial has been terminated due to severe immune related adverse events in five patients. A planned clinical trial for MRX34 for melanoma patients has also been abandoned.

4.3. Viral delivery

Adenovirus, adeno-associated virus (AAV) and lentivirus systems have also been used to deliver miRNAs [81]. AAV vectors tend to be the safest in terms of lessened risk of immune rejection with low host immune response [82]. 80–90% of the human population have AAV seropositivity which accounts for low host immune response, and AAVs have not been disease-associated. AAV vectors are replication deficient and have weak toxicity. They are able to transfect into dividing and non-dividing cells. Disadvantages of AAVs include a packaging capacity of less than 5 kilobases and antibody neutralisation due to prior exposure of humans to different AAV serotypes as well as limited scope for repeat dosing. Nonetheless AAVs have been used for miRNA delivery.

In a mouse model, Kota et al. used the AAV system to deliver miR-26a, showing a suppression of tumourigenesis of hepatocellular carcinoma [81]. It was non-toxic, and a 90% transduction of hepatocytes was shown with fluorescent microscopy. This is in comparison to another murine model where a lentiviral vector was administered intranasally to deliver let-7 to test the effect of let-7 replacement on K-Ras dependent lung tumours [83]. The authors acknowledge that this approach may be limited in the clinical setting, as immunohistochemistry showed poor knockdown of let-7 targets. More recently, the AAV vectors have been further refined into the recombinant AAV (rAAV), that are reportedly more reliable. These rAAVs are being used in miRNA work to deplete target miRNAs and study mRNA functions [84].

4.4. EDV™ miRNA delivery

One other miRNA delivery modality that has entered human trials is the EDV nanocell, (EnGeneIC Dream Vector) developed by EnGeneIC, Australia. This is aimed at overcoming the main problems associated with systemic chemotherapy treatment [85], such as systemic toxicity and side effects without necessarily an efficacious and targeted delivery of treatment to tumour cells. EDVs (nanocells) are anuclear, bacterially derived nanoparticles, of 400 nM diameter. The nanocells can be loaded with chemotherapeutic agents and non-coding RNA molecules, such as miRNAs and siRNAs. Its tumour specificity arises from bispecific antibodies, with one arm attaching to the lipopolysaccharide surface of the nanocell and the other arm recognising a specific cell surface receptor of the target tumour cell, such as epidermal growth factor receptor (EGFR). The size of the EDV allows it to extravasate into the leaky vasculature in the tumour micro-environment, and the bispecific antibody allows for targeted uptake via receptor engagement and micropinocytosis.

EDVs have been loaded with miRNAs and tested in a number of rare tumours in the laboratory. In work done with ACC, Soon et al. identified miR-7 as being 18 fold underexpressed in ACC versus normal adrenal samples [64]. In the same group, Glover et al. [86] undertook systemic replacement of miR-7 via EDVs in a mouse xenograft model. Following the delivery of miR-7 via EDVs in mouse xenograft experiments, tumour reduction was observed in both the commercial ACC cell line H295R and primary patient derived ACC cell line xenografts. Efficient EDV delivery of miR-7 therapy to the xenografts was confirmed via RT-qPCR of the excised tumours. Additionally, it was found that miR-7 targets Raf-1 proto-oncogene (*RAF1*) and mechanistic target of rapamycin (*MTOR*), which were reduced in the xenografts. In malignant mesothelioma, it was shown that by replacing underexpressed miR-16 in preclinical models of mesothelioma, xenograft growth was inhibited [87].

Following these encouraging preclinical *in vivo* studies, one clinical trial of miRNA replacement therapy via EDVs has been

undertaken and completed. In this first-in-man clinical trial, TargomiRs - EDVs loaded with miR-16 based mimic microRNAs and targeted to EGFR – were administered to 26 malignant mesothelioma patients with progressive disease who had failed first and second line treatment with conventional chemotherapy [88]. This study aimed to assess the safety, optimal dosing, and efficacy of TargomiRs in patients with malignant pleural mesothelioma. Of the 26 patients, five experienced dose limiting toxicity, and twenty-two patients were assessed for response to TargomiR treatment via CT. One patient had a dramatic partial response [89], fifteen (68%) had stable disease, and six had progressive disease. The duration of response for the one patient's responsive disease was 32 weeks. Median overall survival was 200 days, and 21 deaths occurred during the trial, of which 20 were related to tumour progression. Overall, the investigators describe the trial as promising, and they anticipate these results will form a basis for further trials with expanded patient cohorts, particularly in combination with chemotherapeutic regimens.

4.5. An example of in vivo miRNA therapy prostate cancer

We have also sought to investigate how miRNA-based therapy could be utilised in common cancers. Prostate cancer imposes a heavy health burden in Australia. The Australian Institute of Health and Wellbeing estimates 16,665 new cases in 2017, comprising 23% of all new male cancer cases, with an estimated 3452 deaths from prostate cancer in 2017. Prostate cancer presents a very good 5-year survival rate, but for metastatic disease, it is less than 30%. Recent studies have also showed miRNAs play an important roles in prostate cancer tumorigenesis [90].

Prostate cancer, amongst other cancers, exhibit loss of heterozygosity (LOH) at the 13q genetic locus and studies have examined the frequency of 13q LOH in prostate cancer, with increased LOH at 13q correlated with higher stage and grade prostate cancers [91]. miR-15a/miR-16 are transcribed in a cluster at the 13q14 locus. In prostate cancer, studies have found a significant 80% reduction in miR-15a/miR-16 levels in Stage 2 and 3 prostate cancers when compared to normal prostate samples [92]. In addition, the expression of the genes *BCL2* (B-cell lymphoma 2), *CCND1* (cyclin D1) and *WNT3A* (Wingless-Type MMTV Integration Site Family, Member 3A) were inversely regulated with miR-15a/miR-16 expression levels. We sought to apply and test these findings in an *in vivo* model. In partnership with EnGeneIC, we tested the effect of miRNA replacement therapy in hormone resistant prostate cancer xenografts by intravenous injection of miR-15a packaged in EDVs.

Prostate cancer cells DU-145 were first used for xenograft implantations, and the xenografts reached 100–130 mm^3 three weeks after the implantations. Mice were randomised to three study groups, being treated with either miR-15a packaged in EDVs ($^{EGFR}EDV_{miR\text{-}15a}$), EDVs containing a scramble miRNA sequence as a negative control ($^{EGFR}EDV_{miR\text{-}NC}$) or saline.

Mice were treated three times per week for two weeks when a significant difference between the treatment and control groups was detected as per the animal ethical protocols (Fig. 1).

We further sought to explore the effect of miR-15a restoration via EDVs on key mRNA targets in the mouse xenografts. In this study, prostate cancer cells PC-3 were utilised and xenografts were harvested after one week's treatments for RNA extraction. Both the molecular targets of miR-15a, *BCL2* and *WNT3A* were significantly down-regulated by over 2-fold (Fig. 2).

5. Conclusions and future directions

The field of miRNA research holds exciting prospects for cancers with unmet clinical needs in terms of diagnostics, prognostics and therapeutics. There has been an evolution from profiling altered miRNA expressions in cancers to clinical trials with miRNAs as therapy, in the last decade. We have shown its promise with miRNA replacement therapy in both pre-clinical and clinical pilot studies and its potential use in personalised medicine.

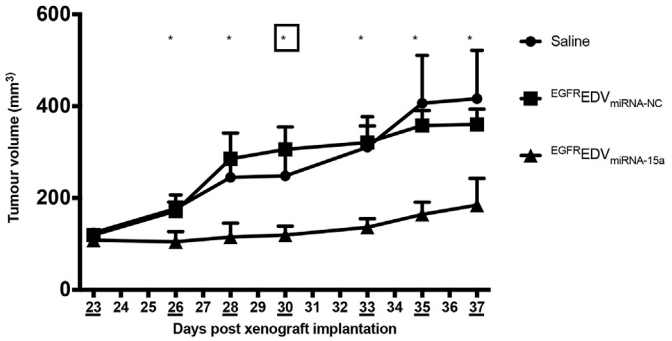

Fig. 1. Targeted miR-15a therapy effect on DU-145 xenografts. Targeted miR-15a replacement therapy via EDVs virtually arrests and stabilises prostate cancer xenograft growth. Mean volumes shown for each group. n = 5. Error bars represent the standard error of the mean (SEM). The underscored "days post xenograft treatment" represent the days of EDV/saline treatment. Asterisked time points represent an observed statistically significant tumour volume difference between the $^{EGFR}EDV_{miR\text{-}15a}$ and $^{EGFR}EDV_{miR\text{-}NC}$ groups. The maximal mean difference in tumour volume between the two treatment groups was 61% on Day 30 (denoted by the boxed asterisk).

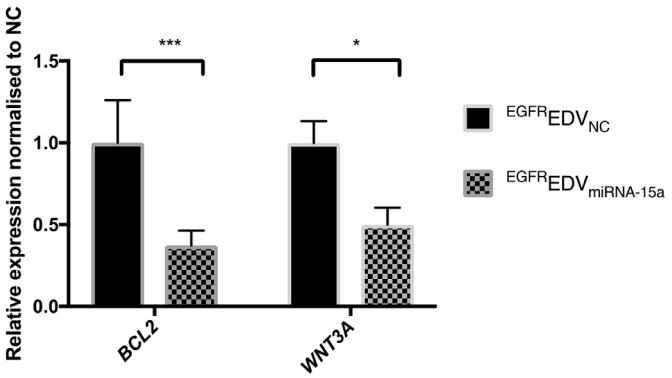

Fig. 2. miR-15a effected target knockdown in PC3 xenograft. Systemic miR-15a treatment via EDVs significantly enabled knockdown of mRNA targets. n = 6 for each treatment group; *GAPDH* was the reference gene for RT-qPCR, error bars show SEM. *Denotes p = 0.03*** denotes p = 0.008.

Declarations of interest

H. Brahmbhatt & J.A. MacDiarmid have ownership interests (including patents) in EnGeneIC Pty Ltd. J. Weiss & N. Mugridge are employees of EnGeneIC Pty Ltd. S.B Sidhu has ownership interests in EnGeneIC Pty Ltd. The other authors declare no conflict of interest.

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