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Isolation of Secreted MicroRNAs (miRNAs) from Cell-Conditioned Media

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Title: Isolation of secreted microRNAs (miRNAs) from cell-conditioned media

Running Title: In-vitro secretion of microRNAs

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Abstract

MicroRNAs (miRNAs) have been found to be stable in the circulation of cancer patients raising their potential as circulating biomarkers of disease. The specific source and role, however, of miRNAs in the circulation is unknown and requires elucidation to determine their true potential. In this study, along with primary tissue explants and primary stromal cells, three breast cancer cell lines were employed, including T47D, MDA-MB-231 and SK-BR-3. Tissue explants were harvested in theatre, with informed patient consent, and included tumour, tumour associated normal, and diseased lymph node samples. Cell-conditioned media containing all factors secreted by the cells were harvested. MiRNAs were extracted from samples using 5 different extraction techniques including the blood protocol, RNeasy® (Qiagen), miRNeasy® mini kit (Qiagen), mirVana™ isolation kit (Ambion) and RNAqueous® kit (Ambion). MiRNAs were successfully isolated from all media samples collected from cell lines, primary cells and fresh tissue explants. However, there was remarkable variation in yield depending on the extraction method used. Aliquots of the same samples were extracted, revealing the two column extraction protocol of the mirVana® miRNA isolation kit to be the most suitable approach. A range of miRNAs, including miR-16, miR-195, miR-497 and miR-10b, were successfully amplified. While miR-16 and miR-195 were detected in media from both cell lines and tissue explants, miR-497 and miR-10b were only detected in secretions from whole tissue explants. The ability to achieve reliable and reproducible miRNA yields from cell-conditioned media is vital for the successful amplification of miRNAs by RQ-PCR.

Key Words:

Biomarker; breast cancer; cell culture media; circulation; extraction; microRNA; secretion.

Introduction

MicroRNAs (miRNAs) are a class of small (18-22 nucleotides), non-coding RNA molecules found in plants, animals, and DNA viruses which often act to inhibit gene expression post-transcriptionally. This is achieved by binding to specific messenger RNA (mRNA) targets and promoting their degradation and/or translational inhibition [1]. Hundreds of mRNAs may be regulated by only one miRNA and therefore they can exert their influences on a wide variety of gene expression networks [2]. MiRNAs play a vital role in the regulation of crucial biological and pathological processes, most notably in development, aging, cancer and neurological disease development and progression [3]. Indeed, it may prove difficult to find a fundamental biological process which miRNAs do not play a role in, with over half of mammalian messages under selective pressure to maintain pairing to miRNAs [4, 5]. As a result, miRNAs are being investigated as potential biomarkers which could be utilized in a wide variety of molecular applications, including the diagnosis of cancer, cardiovascular and auto-immune diseases [2, 6, 7]. The discovery that miRNAs are frequently up-regulated or down-regulated in tumours when compared to normal tissue supports their role as either oncomirs or tumour suppressors [6, 8, 9]. Initially, the majority of research focused on the role of miRNAs in tissue, however, more recently it has been discovered that miRNAs are in fact detectable in the circulation [10-15].

The use of circulating microRNAs as potential biomarkers of disease is a rapidly evolving field of study. This hot topic has initiated widespread study of the abundance, half-life and stability of miRNAs in the circulation, which require careful consideration if they are to be implemented in the clinical setting [12]. Numerous studies have been performed assessing the effect of specimen type, the presence of cellular contaminants and stability [10, 16-18]. Mitchell et al [15] first reported the presence of miRNAs in the circulation in a remarkably stable form, while subsequent studies have also found circulating miRNAs to be significantly more stable than their protein counterparts during long term storage [18, 19]. It has been suggested that dysregulation of some miRNAs may be disease specific, for example, in the context of breast cancer, Heneghan et al [20] reported that circulating miR-195 was significantly elevated only in breast cancer patients and not those with other tumour types.

In recent years, there has been an explosion of interest in miRNAs as potential biomarkers of disease, particularly with regards to their presence in the circulation [10, 13, 21]. Novel circulating biomarkers could improve the early

detection, diagnosis and ultimately the clinical management of cancer [22]. However, the true source of miRNAs in the circulation and their exact secretory mechanism is relatively unknown [23]. A number of hypotheses have been put forward. Chin and Slack [11] suggested two possible pathways: 1. tumour miRNAs are present as a result of cell death and lysis, and 2. miRNAs are present as a result of being actively secreted by cells [11]. In terms of active secretion, more recent studies have suggested that this may involve encapsulation of miRNAs into microvesicles, including exosomes and shedding vesicles [24-26]. Alternatively, active secretion could be achieved using a microvesicle-free, RNA binding, protein-dependent pathway [27]. Microvesicles, and indeed exosomes, have been heralded as novel regulators of cell to cell communication, and thus could also provide a mechanism of miRNA transfer between cells [28-30]. An accumulating body of evidence suggests that extracellular vesicles such as exosomes have the ability to be secreted by donor cells and are taken up by acceptor cells [26, 31, 32]. This potential exchange of miRNAs is an exciting and novel dimension to the regulation of a cells phenotype, and is particularly important in cancer [33]. MiRNAs offer immense potential as circulating biomarkers of disease, however, it is essential that we firstly understand their true source and role in disease.

There have been relatively few published reports on the isolation of miRNAs from cell-conditioned media [34, 35]. In addition, there is currently no commercially available extraction kit/technique designed specifically for miRNA isolation from cell-conditioned media. Two research groups have reported successful isolation of miRNAs from the media of four cell lines [36, 37]. Kosaka et al [36] reported miRNA isolation from the cell-conditioned media of HEK293T cells and COS-7 cells using the mirVana™ miRNA isolation kit (Ambion). Turchinovich et al [37] reported use of the miRNeasy® micro Kit (Qiagen) with cell-conditioned media from HEK293T cells and two breast cancer lines, MCF7 and MDA-MB-231. However, it is worth noting the latter group drew attention to the very low yields which they were obtaining [37]. There are no published reports describing analysis of miRNA secretion from tumour tissue explants. Successful RNA analysis relies heavily on the yield, purity and integrity of the extracted RNA. Therefore, an optimum extraction protocol is essential.

The aim of this study was to identify the most reliable and reproducible method for the isolation of miRNAs from cell-conditioned media, utilizing both commercially available cell lines and tumour tissue harvested during surgery. The reproducibility and efficiency of 5 methods/kits were tested and compared. A prerequisite for successful amplification using RQ-PCR is an optimal microRNA yield, therefore an efficient method for RNA extraction is

critical. A panel of four candidate microRNAs (miR-16, miR-195, miR-497 and miR-10b) were chosen for amplification by RQ-PCR in cell-conditioned media samples as these miRNAs have previously been implicated in the circulation of breast cancer patients [20, 38].

Materials and Methods

Cell Culture

The following breast cancer cell lines had previously been purchased from the American Type Culture Collection (ATCC): T47D, MDA-MB-231 and SK-BR-3. Cells were cultured at 37°C, 5% CO₂, with a media change twice weekly and passage every 7 days. The media for each cell line was as follows: RPMI-1640 (T47D), Leibovitz-15 (MDA-MB-231) and McCoy's 5A (SK-BR-3), each supplemented with 10% Foetal Bovine Serum (FBS), and 100IU/ml penicillin G/100 mg/ml streptomycin sulfate (Pen/Strep).

Tissue Explants

Following ethical approval from Galway University Hospitals (GUH) and written informed patient consent, tumour tissue harvested in theatre was weighed and placed into 2mls of culture media. The culture media employed consisted of DMEM +Glutamax supplemented with 10% FBS, and 100IU/ml Pen/Strep. These primary tissue samples included Tumour, Tumour Associated Normal (TAN), and diseased lymph node tissue. TAN refers to tissue at least 2cm from the site of the primary tumour.

Collection of cell-conditioned media

Tissues and cells were incubated in the appropriate media for a period of 24 hours at 37°C, 5% CO₂. The conditioned media (CM), containing all factors secreted by the cell lines and tissues, were then harvested from the cultures, centrifuged for 4 minutes at 201 x g RCFMax (1,000rpm Eppendorf 5810R, A-4-62) at 4°C, and stored at -20°C until required for extraction.

miRNA Isolation

This study investigated the use of five available and widely used miRNA isolation techniques, namely the blood protocol [20], RNeasy® (Qiagen), miRNeasy® mini kit (Qiagen), mirVana™ isolation kit (Ambion) and RNAqueous® kit (Ambion), Table 1.

Method	Source	Design Specification
Blood Protocol [20]	Discipline of Surgery adaption of the TRI Reagent® technique (Molecular Research Center, Inc., Cincinnati, OH) [20]	Utilizes Trizol® reagent and Bromoanisole.
RNeasy®	Qiagen	On-column method designed to isolate total RNA (i.e. small and large)
miRNeasy®	Qiagen	On-column method designed to specifically isolate microRNA
mirVana™	Ambion	Double column method designed to isolate both small and large RNA
RNAqueous®	Ambion	Phenol free method designed for total RNA isolation

Table 1. Methods of extraction used for isolation of miRNAs from cell-conditioned media.

The blood protocol was developed in-house in the Discipline of Surgery, NUI Galway, and is a modified Trizol™ co-purification technique [20]. Previous studies have found this method to provide reliable miRNA yields from whole blood, plasma and serum [20, 39]. For each 1 ml of cell-conditioned media, phase separation was performed by the addition of 3 ml of Trizol®. 200 µl of 1-bromo-4 methoxybenzene was then added to augment the RNA phase separation process. Samples were then centrifuged at 4°C, 15,300 x g RCFMax (14,000rpm Eppendorf 5417R, F45-30-11) for 15 minutes. Total RNA was precipitated using isopropanol and washed with 75% ethanol prior to solubilisation with 60 µl of nuclease free water. Thus, each 1 ml of sample yielded 60 µl of total RNA solution, which was stored at -80°C.

The remaining four miRNA isolation techniques included in this study (Table 1.) all employed commercially available kits which are broadly based on a column/filter isolation method.

Prior to extraction using the RNeasy®, miRNeasy®, mirVana™ and RNAqueous® isolation kits, the following steps were carried out to permit isolation of miRNA from cell-conditioned media:

1. Extractions were performed on 1ml of cell-conditioned media. 700µl of Trizol™ reagent was added to the sample and vortexed. The sample was then left to stand for 5 minutes at room temperature.
2. 140µl of chloroform was added and the sample shaken vigorously for 15 seconds.
3. Samples were centrifuged at 15,300 x g RCFMax (14,000rpm Eppendorf 5417R, F45-30-11), at 4°C, for 15 minutes.
4. The upper aqueous phase was transferred to a new tube, carefully avoiding the interphase.
5. The manufacturer's protocol for each kit was then followed from the separation step.

The RNeasy and miRNeasy Mini Kit (Qiagen®) both combine phenol/guanidine-based lysis of samples and a silica membrane column based purification. Following the steps described previously, the upper, aqueous phase was extracted, and ethanol was added to provide appropriate binding conditions for all RNA molecules from 18 nucleotides (nt) upwards. The sample was then applied to the RNeasy/miRNeasy Mini spin column, where the total RNA binds to the silica membrane and phenol and other contaminants are efficiently washed away. High quality RNA is then eluted in RNase-free water.

The RNAqueous® Kit (Ambion®) is designed for phenol-free total RNA isolation using a guanidinium-based lysis/denaturant and Glass Fiber Filter (GFF) separation technology. The RNAqueous method is based on the ability of glass fibers to bind nucleic acids in concentrated chaotropic salt solutions. Following the steps described previously, the lysate was diluted with an ethanol solution to make the RNA competent for binding to the GFF in the filter cartridge. This solution was passed through the filter pad where RNA binds and most other cellular contents flow through. The filter cartridge was washed 3 times to remove contaminants, and the RNA was eluted in RNase-free water.

The mirVana™ miRNA Isolation Kit (Ambion®) employs organic extraction, as described previously followed by purification on two sequential Glass Fibre Filters (GFF) under specialized binding and wash conditions. Unlike other methods, the mirVana™ kit utilizes two sequential GFFs, as it has been suggested that the small RNAs are essentially lost in the first filtration through the column and therefore a second filtration is required to capture the tiny microRNAs.

The concentration and purity of miRNA was assessed using NanoDrop™ 1000 spectrophotometer (Nanodrop Technologies, Willmington, DE, USA). The wavelength dependent extinction coefficient “33” was taken to represent the microcomponent of all RNA in solution.

Amplification of miRNAs

For each sample, 100ng of miRNA was reverse transcribed into cDNA using MultiScribe™ reverse transcriptase (Applied Biosystems, Foster City, CA, USA) and sequence-specific stem-loop primers (Applied Biosystems) which target the mature miRNA sequence. RQ-PCR was performed using TaqMan® probes (Applied Biosystems) specific for the miRNAs of interest, miR-16, miR-195, miR-497 and miR-10b. RQ-PCR was performed on a 7900 HT Fast Real-Time PCR System (Applied Biosystems). An Inter Assay Control (IAC), Reverse Transcription (RT) blank and a No Template Control (NTC) were included on each plate. All reactions were performed in triplicate. The threshold standard deviation accepted for intra- and inter-assay replicates was <0.3.

Results and Discussion

A wide variety of sample types were extracted using the different extraction techniques. This included breast cancer cell lines (T47D, SK-BR-3, MDA-MB-231), tissue explants (tumour, tumour associated normal (TAN) and diseased lymph node) and primary cell populations e.g. stromal cells. A total of n=90 samples were extracted using each technique. A yield of >20ng/μl was required for RQ-PCR amplification and therefore anything obtained less than this was not considered sufficient. Only 20 out of the 90 samples yielded sufficient miRNA for progression. Examples of yields obtained from individual samples can be seen in Table 2. The results were widely variable with typically the highest yields obtained from tissue explants, which is to be expected due to cellularity, with the highest yield obtained from diseased tumour lymph node tissue. It is worth noting, however, that miRNAs were also detected in the cell-conditioned media of breast cancer cell lines and primary stromal cells. Of the top 10 yields obtained from all samples, 8 of these were obtained using the mirVana™ miRNA isolation kit. The blood protocol and RNeasy® miRNA isolation techniques were found to be the least successful for the isolation of miRNA from cell-conditioned media.

Sample Identifier	Yield ng/μl
Lymph node Explant A	232.43
SK-BR-3	65.7
Tumour Explant A	64.9
T47D	63.2
Primary stromal cells	42.85
Tumour Explant B	37.12
Lymph node Explant A	32.7
MDA-MB-231	31.52
Tumour Explant	27.86
TAN Explant	24.24
SK-BR-3	21.87

Table 2. MiRNA yield from conditioned media harvested from breast cancer cell lines and tissue explants.

This suggested that the mirVana™ isolation kit may be optimal for miRNA extraction from cell-conditioned media. A direct comparison of methods was then performed using the mirVana™, miRNeasy® and RNAqueous® isolation techniques on aliquots of the same samples. Here, 3 aliquots of the same samples were extracted using each of the 3 extraction techniques. There was significant variability of results obtained using the different extraction techniques, even from within the same sample, which can be seen in Table 3.

Sample Identifier	Method of Extraction	Yield ng/μl
T47D	mirVana™	63.2
	miRNeasy®	4.59
	RNAqueous®	12.92
MDA-MB-231	mirVana™	31.52
	miRNeasy®	4.17
	RNAqueous®	18.68
SK-BR-3	mirVana™	65.7
	miRNeasy®	4.61
	RNAqueous®	12.68

Table 3. MiRNA yields obtained from aliquots of the same samples using the mirVana™, miRNeasy® and RNAqueous® techniques.

The results showed that consistent reproducible results were not obtained using the miRNeasy® or RNAqueous® techniques. In all cases, the mirVana™ miRNA isolation kit provided consistently high and reproducible yields for the successful isolation of miRNAs from cell-conditioned media (e.g. 2-4 fold increase in yield with mirVana™ kit). A range of miRNAs, including miR-16, miR-195, miR-497 and miR-10b, were successfully amplified from each of these samples using RQ-PCR (Table 4.), confirming the presence of intact miRNAs.

Sample	miR-16	miR-195	miR-497	miR-10b
T47D	+	+	-	-
MDA-MB-231	+	+	-	-
SK-BR-3	+	+	-	-
Tumour Explant	+	+	+	+
TAN Explant	+	+	+	+

Table 4. Amplification of miR-16, miR-195, miR-497 and miR-10b in cell-conditioned media samples from breast cancer cell lines and tissue explants ('+' denotes detected, '-' denotes not detected).

While miR-16 and miR-195 were detectable in all cell-conditioned media samples collected from both fresh tissue explants and breast cancer cell lines, miR-497 and miR-10b were only detectable in media exposed to the fresh tissue explants. This suggests that other cellular components of the tumour may be the source of the miRNAs and not just the epithelial cells alone. This is a very interesting area and does require further investigation. The stability, and indeed the persistence of action, of miRNAs is a biophysical parameter which warrants careful consideration. Generally, miRNAs are considered to be relatively stable with long half lives of approximately 2 weeks [15]. However, some studies have shown selected miRNAs to have relatively short half lives (~1-3.5hrs), with several brain abundant miRNAs shown to be surprisingly restricted [40]. In this current study, all of the cell-conditioned media samples were collected in the same way and were frozen at -20°C within 20 minutes of harvest. One would expect that if half life were the issue, then the miRNAs of interest would not be detectable in any of the samples collected.

Conclusion

The successful isolation of intact miRNAs from cell conditioned media in an in vitro setting is a prerequisite for expression analyses of secreted miRNAs. This study found the mirVana™ miRNA isolation kit to obtain the most reproducible and consistently high yields when compared to other methods available. The molecular mechanisms which drive this secretion have yet to be elucidated. The use of an optimal method for isolating these secreted miRNAs will support elucidation of the mechanism of secretion, the true cellular source and role of these molecules in the circulation.

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