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REVIEW

The choice of endogenous controls in exosomal microRNA assessments from biofluids

G. Occhipinti[1] · M. Giulietti[1] · G. Principato[1] · F. Piva[1]

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Abstract The assessment of differentially expressed microRNAs in patients and healthy controls is important to identify potential tumor biomarkers. Recently, it has been shown that the microRNA levels in exosomes are more correlated with the clinical-pathological variables than vesicle-free microRNAs (miRNAs) in biofluids; therefore, there is an increasing interest in these specific evaluations. However, these measurements can be affected by experimental problems that not always are evaluated and/or by inadequate procedural choices. In particular, exosome isolation and miRNA extraction procedures are crucial to avoid contaminations, and even the choice of the most suitable purity controls is important. Moreover, a stable endogenous RNA should be used for normalization of miRNA expression obtained by reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR) in order to make these measures comparable among different samples. A rushed choice of the endogenous control can bias study conclusions without revealing inconsistencies. Unfortunately, a few studies systematically identified the best normalizer for their specific experimental context. Instead, sometimes, the normalization procedures were performed in a disputable way or the normalizer choices simply based on the previous literature. Here, we reviewed the studies where the exosomal miRNA profiling was assessed in human biofluids to point out the adopted procedures and the specific endogenous controls chosen for normalization.

Keywords miRNA · Exosomes · Profiling · Normalization · Endogenous control

✉ F. Piva
f.piva@univpm.it

[1] Department of Specialistic Clinical and Odontostomatological Sciences, Polytechnic University of Marche, Via Brecce Bianche, 60131 Ancona, Italy

Introduction

MicroRNAs are small noncoding RNAs of about 22 nucleotides that are implicated in post-transcriptional gene regulatory mechanisms. They bind the complementary 3′ untranslated or open reading frame regions of target messenger RNAs (mRNAs) causing their degradation or the downregulation of protein expression [1], thus controlling various biological processes. MicroRNAs (miRNAs) are present within the cells but can be also secreted by them into the extracellular environment in order to reach specific target cells and modify their behavior. miRNAs that have been released in body fluids maintain their functionality since they are protected from degradation of endogenous RNases by the Ago2 multiprotein complexes [2] or high-density lipoproteins [3] or enclosed in microvesicles and exosomes [4, 5]. The latter mechanism is important since it allows the RNA to be delivered to specific target cells.

Exosomes are small vesicles of about 30–140 nm in diameter that are secreted from cells by exocytosis after fusion of multivesicular bodies with plasma membrane [6] and are involved in communication among cells both in physiological and in pathological conditions. Exosomes carry out horizontal information transfer by delivering their cargo, constituted by miRNAs, proteins, lipids, and other nucleic acids. It has been demonstrated that only some miRNAs can be loaded into exosomes; moreover, they are dynamically sorted depending

on the necessity of the sender cell, so miRNA type and quantity in exosomes provide useful information about the pathophysiological state of a tissue [7]. The sorting seems to be regulated by specific RNA sequence motifs [8, 9] that constitute an export language, so this mechanism reminds of the nucleo-cytoplasmic export of the RNAs [10].

Many studies in recent years highlighted that exosomes secreted by tumor cells presented specific over-expressed miRNAs, which are associated with the immunosuppression of antitumor responses or tumor growth and metastasis progression or with the transmission of drug resistance to sensitive cells [11, 12]. Moreover, it has been demonstrated that miRNAs in exosomes are involved also in neuronal-signaling pathways, so they could be employed for the prediction and monitoring of neuronal diseases like Alzheimer [13]. Exosomal miRNAs extracted from urine could be informative also for various kidney disorders [14, 15]. They are important in the assisted reproductive field since it has been shown that exosomal miRNAs in follicular fluid could be an indicator of oocyte quality [16].

For these reasons, miRNAs contained in exosomes are considered important biomarkers that could be easily detected in a noninvasive way from all body fluids [17], so it is important to be able to accurately quantify miRNA expression in order to compare the miRNA profiling in patients and in healthy controls. Usually, reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR) is the method that, easily and with high accuracy, provides miRNA dosage from biological fluids [18]. In order to make the measures comparable among different samples, it seems natural to start with the same volumes of biological fluid for each sample but this does not solve the problem due to the different concentrations of exosomes in different samples.

Quantification of exosomes from biofluids

Measuring the concentration of exosomes in a sample is not banal, since it is not possible to accurately count these vesicles due to their small size which prevents the flow cytofluorimeter from directly seeing them. Exosomes can be coarsely quantified by the Bradford assay that measures their whole protein content [19], but it has the disadvantage of not distinguishing the protein content deriving from cellular contamination which is always present, to some extent, in an extraction. Purified exosomes can be quantified using nanoparticle tracking analysis (NTA) that measures size distribution and estimates the concentration of cellular vesicles, by analyzing a captured video of the light scattering produced by the particles moving under Brownian motion in liquid suspension [20]. This method does not execute a count of single exosomes and does not distinguish if a particle in the size range of 30–140 nm is an exosome or a small debris. Enzyme-linked immunosorbent assay (ELISA) is a quantitative technique, highly specific thanks to antibodies, and highly sensitive thanks to signal amplification methods that can be used to measure the concentration of exosomes in a sample. It can be used also with dirty matrices since there is a phase of exosome immunocapture in which ubiquitous and specific exosomal proteins are exploited. Unfortunately, among the disadvantages of the ELISA, it should be taken into account that accurate measures can be affected by antibody quality and coating, blocking, and incubation procedures.

However, none of these methods is able to measure the exosome concentration with high accuracy. A molecule which is ubiquitous and specific of the exosomes and could be measured with highly sensitive and accurate techniques such as RT-qPCR would be ideal.

Extraction yields

Despite the same starting volumes of biological fluid for each sample, the upstream RT-qPCR procedures, like sample preparation, exosome isolation, and miRNA extraction, provide different yields that do not make the miRNA levels comparable among samples.

To limit these problems, the use of a standardized method of sample collection and storage is very important. It is essential that the taking of the samples is performed in all individuals of the study at the same time of the day, since even fasting or postprandial status can influence the extracellular vesicle production by cells [21]. Also, the extraction of plasma or serum from blood should be done after the same short period of time from blood withdrawal and using the identical rotor angle of the centrifuge. This is because blood cells release exosomes during blood storage, thus altering the populations of exosomes that will be collected by plasma/serum.

An ideal molecule should also overcome the problem of the different extraction yields as it could be used to normalize the levels of miRNAs assessed in the samples.

Cellular and extracellular contamination

During the procedures of exosomal miRNA extraction from biofluids, it is possible that cellular RNAs contaminate the preparations causing an alteration of the miRNA profiles. In order to measure only exosomal miRNAs, the presence of cellular RNAs should be evaluated or prevented.

Tokuhisa et al. [22], by using Agilent 2100 Bioanalyzer, assessed cellular RNA contamination in their samples by measuring the 18S and 28S ribosomal RNA peaks from the exosomal fraction of malignant ascites and peritoneal lavage fluid indicating that it was not contaminated with intracellular RNA. In a work aimed to establish a standard procedure for exosome purification and their miRNA extraction from urine samples comparing six different methods, Agilent Bioanalyzer showed that in all the obtained exosomal RNA

preparations, there was the absence of ribosomal RNAs and that miRNAs were the most abundant RNA species [23]. So the high quality of these preparations has allowed to perform the following quantitative measures in order to compare them and to propose the best RNA isolation method [23].

In order to detect RNA of strictly exosomal origin, RNase A treatment was used after exosome isolation from saliva to degrade residual cellular RNAs [24]. In fact, the RNase A treatment (100 ng/ml at 37 °C for 10 or 20 min) did not degrade RNA within exosomes in plasma and urine samples [14, 25] that therefore represent transport vehicles resistant to RNase A treatment. However, it was demonstrated that exosomal miRNAs isolated from HT-29 cell medium heavily treated with RNase (5 μg/ml at 37 °C for 30 min) were only partially protected by exosomes [26]. Therefore, the side effects of the prolonged exposition and high concentration of RNAse A should be taken into account by researchers.

Another, recently discovered, source of contamination is constituted by lipoprotein-bound miRNAs [27]. Operators have to consider that high-density (HDL) and low-density lipoproteins (LDL) are always present in exosomal extracts, except when performed by immune-precipitation techniques. Since it has not yet been demonstrated if miRNAs are enclosed or externally associated with lipoproteins [28], RNase A treatment could not completely remove this source of contamination.

Hemolysis interference

In plasma or serum samples, the quantification of exosomal miRNAs can be impaired also by hemolysis that causes contamination with erythrocyte-derived miRNAs. In fact, it was demonstrated that miR-486-5p, miR-451, miR-92a, and miR-16 are enriched in red blood cells and their expression significantly increases in hemolyzed samples [29, 30]. It was also observed that exosomal RNA extracted from plasma samples using an exosome isolation kit was enriched in tRNA, a cellular contamination index [25].

Surprisingly, hemolysis provides an effective protection of free circulating miRNAs from plasma RNases since blood cells release RNase inhibitors [31]. Therefore, it is suggested to treat the plasma samples with RNase A and, in case of hemolysis, reinforce this treatment to exceed the effect of endogenous inhibitor.

A method of identifying the presence of hemolysis in total plasma is to measure the absorbance of the oxy-hemoglobin at $\lambda = 414$ nm, but it is not a reliable method because the lipid content in plasma could determine a false positive [32]. However, some authors resolved the problem normalizing with the absorbance measurements at 375 [33] or 385 nm [32] which are indicators of lipemia.

If a sample is contaminated by hemolysis, it should not necessarily be rejected but assessed to see if this hitch causes differences in the miRNA concentration between a hemolyzed and non-hemolyzed aliquots from the same sample [32].

RT-qPCR normalization

In Table 1, we show that few works have performed a systematical search for exosomal miRNAs as the ideal molecules for RT-qPCR normalization and there are no endogenous ones which can be used in all situations. Other studies, instead, chose the normalizer based on previous literature, and sometimes, this normalizer was validated again on the specific samples under investigation (Table 2). It can be noted that small nuclear RNA (snRNA) U6 and miR-16 were frequently used in studies of exosomal miRNA profiling from different body fluids often without any validation of their expression stability. In particular, Table 2 shows that sometimes snRNA U6 is used to normalize the expression level of circulating exosomal miRNAs in blood and urine samples [34–36]. However, in serum exosomal fraction of patients with hepatitis B, hepatocellular carcinoma, and healthy volunteers, snRNA U6 had a high interindividual variability [37], as well as in whole serum samples from healthy people, liver fibrosis, and intensive care unit patients [38]. Moreover, it should be noted that although some papers showed that snRNA U6 is present also in exosomes [39, 40], it still remains controversial if this could be due to cellular contamination. In fact, snRNA U6 is exclusively localized within the nucleus and not in the cytoplasm from where RNAs are loaded into exosomes [41–43].

Regarding miR-16, it was used as a normalizer in works that studied exosomal miRNAs of saliva [44] and serum [45] but without testing its stability. Performing this assessment would have been very important, because some works showed contradictory results regarding miR-16 expression stability. In particular, miR-16 in exosomes from malignant ascites and peritoneal lavage fluids was consistently expressed and thus used as potentially endogenous control [22]. Also in exosomes from serum of healthy individuals and breast cancer patients, both miR-16 and miR-484 have been shown to be good reference miRNAs [46]. On the contrary, miR-16 was highly unstable in exosomal serum from patients with hepatitis B, hepatocellular carcinoma, and healthy subjects [37]. Other authors showed that miR-16 and miR-451 had constant levels in non-hemolyzed total plasma samples but when they were released by red blood cells, due to hemolysis, their levels were variable [30]. Exosomal miR-451 was the most stable miRNA also in non-hemolyzed serum samples of healthy controls and mild cognitive impairment and Alzheimer's disease patients [13].

In a study where the exosomal level of miR-21 was compared among cerebrospinal fluid samples and serum samples from glioma patients and non-tumor controls, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used for the

Table 1 Assessment of best endogenous exosomal RNAs

Algorithms /methods	Identified endogenous controls	Disease status	Biofluids	References
NormFinder and BestKeeper	snRNA U6 and HY3, snRNA U48 and HY3	Hematopoietic stem cell transplantation and healthy	Serum urine	Crossland et al. [58]
GeNorm, NormFinder and BestKeeper	miR-221, let-7a, miR-26a	Hepatocellular carcinoma	Serum	Li et al. [60]
Global normalization method	miR-320, snRNA U6	Uveal melanoma, cornea donors, and healthy serum donors	Vitreal humor and serum	Ragusa et al. [67]
GeNorm and NormFinder	miR-221, miR-103, let-7a, miR-181c, miR-181a, miR-26a	Hepatitis B, hepatocellular carcinoma, and healthy subjects	Serum	Li et al. [37]
GeNorm and DataAssist	miR-451	Alzheimer's disease, mild cognitive impairment, and healthy controls	Serum	Cheng et al. [13]
GeNorm and DataAssist	miR-126, miR-28-3p, miR-145	Healthy women	Follicular fluid and plasma	Santonocito et al. [16]
NormFinder and BestKeeper	miR-30a-5p, miR-30e-5p	Colorectal cancer, pancreatic cancer, prostate cancer, and healthy individuals	Plasma	Huang et al. [59]

In particular, we showed the algorithms and methods used to process the raw expression data in order to give back the most stable RNAs

normalization of miR-21 data [47]. Also for GAPDH mRNA, even though it was detected in exosomes [48–50], a doubt remains that its detection could be due to cellular contamination, which is always potentially present when ultracentrifugation and precipitation reagents are used for exosome isolation. However, the usage of GAPDH is unadvisable since it is better to normalize miRNA values with a molecule of the same kind. For example, in another work, exosomal GAPDH was correctly used to normalize exosomal mRNAs [50].

MiR-642a-3p was chosen as endogenous control using the microarray analysis, and further validation was carried out in serum exosomes of patients with mild and extremely severe hand, foot, and mouth disease and healthy groups [51]. Also miR-451 resulted a valid endogenous control by using the previous methods in serum exosomes of colorectal cancer patients and healthy controls [52].

Identification of endogenous controls

The works focusing on the identification of reliable endogenous controls adopted two or more algorithms such as geNorm, NormFinder, BestKeeper, and DataAssist that calculate the most stable miRNAs among a set of candidates (Tab 1). The geNorm algorithm reveals the most stable reference genes using the geometric mean of the selected genes as a normalization factor [53]. This tool calculates the pairwise variation (V) for each miRNA with all other miRNAs, and for each miRNAs gives back an index (M) which is the average of its Vs. The most stable miRNAs have the minor M values [54]. GeNorm was used to identify the best endogenous miRNA on deep sequencing data obtained from a complementary DNA (cDNA) library of exosomal RNA extracted from serum of healthy controls, mild cognitive impairment participants, and patients with Alzheimer's disease [13]. miR-451 resulted as the most stable endogenous control identified by this tool, and it was used together with the exogenous control (Cel-miR-39) to calculate the ΔCt using the DataAssist software [13].

DataAssist is a tool developed to analyze a large collection of gene expression values from TaqMan assays [55]. It allows to calculate relative quantification through the comparative ΔCt method and to measure gene stability using the geNorm algorithm in order to select the controls for data normalization.

NormFinder tool identifies the optimal normalization genes considering that they are usually grouped in sample groups (e.g., normal and pathological); therefore, it assesses both the intergroup and intragroup variations of gene expression [56].

GeNorm and NormFinder were used to find the best endogenous miRNAs in a study on serum exosomal samples between hepatitis B or hepatocellular carcinoma patients versus healthy individuals. miR-221, miR-103, let-7a, miR-181c, miR-181a, and miR-26a resulted as the most stable endogenous controls while miR-16, miR-22, and U6 were not reliable as internal controls [37].

BestKeeper tool calculates the expression stability of miRNA candidates processing the standard deviation and coefficient of variance, the geometric means, and the pairwise correlations of the Ct values [57].

NormFinder and BestKeeper were applied to assess the best endogenous controls in samples of extracellular vesicles isolated from sera and urine of healthy controls and hematopoietic stem cell transplantation patients, highlighting HY3, snRNA U6, and snRNA U48 [58].

In samples of exosomal RNAs from plasma of 192 individuals (50 healthy individuals, 100 colorectal cancer patients,

Table 2 Endogenous controls used to normalize expression data of circulating exosomal miRNAs

Endogenous control	Condition	Biofluid/tissue	Authors
miR-191-5p, snRNA U6	Prostate cancer	Urine	Samsonov et al. [35]
snRNA U44, snRNA U6	Parkinson and Alzheimer	Cerebrospinal fluid	Gui et al. [78]
miR-16	Lichen planus	Saliva	Byun et al. [44]
miR-4739	Aging	Saliva	Machida et al. [79]
GAPDH	Glioma	Blood and CSF and cell culture medium	Shi et al. [47]
miR-16	Gastric cancer	Malignant ascites and peritoneal lavage fluid	Tokuhisa et al. [22]
miR-16a	Human colorectal cancer	Serum	Matsumura et al. [45]
snRNA U6	Adenocarcinoma of esophagus	Serum	Warnecke-Eberz et al. [34]
miR-16, miR-484	Breast cancer	Serum	Eichelser et al. [46]
miR-642a-3p	Hand, foot, and mouth disease (HFMD)	Serum	Jia et al. [51]
snRNA U6	Laryngeal squamous cell carcinoma	Serum	Wang J et al. [36]
snRNA U6	Hepatocellular carcinoma	Serum	Wang H et al. [80]
miR-451	Colon cancer	Serum	Ogata-Kawata et al. [52]
miR-16	Stability under various storage conditions	Plasma	Ge et al. [81]
snRNA U6	Diabetic nephropathy, focal segmental glomerulosclerosis, IgA nephropathy	Urine	Lv et al. [14]
snRNA U6	Diabetic nephropathy	Urine	Barutta et al. [82]
Small nucleolar RNAs	Lung cancer before and after surgery	Plasma	Aushev et al. [83]
snRNA U6	Pancreatic cancer	Serum	Que et al. [84]
snRNA U6	Renal fibrosis/chronic kidney disease	Urine	Lv et al. [15]
snRNA U6	Novel miRNAs identification	Breast milk	Munch et al. [85]
miR-423	Schizophrenia and bipolar disorder	Postmortem brain tissue	Benigan et al. [86]
miR-16	Esophageal squamous cell carcinoma	Serum	Tanaka et al. [87]
snRNA U48	Sjögren's syndrome	Saliva	Michael et al. [24]

6 pancreatic cancer patients, and 36 PCa patients), NormFinder and BestKeeper processing and RT-qPCR validations give back miR-30a-5p and miR-30e-5p for their better stability [59].

GeNorm, NormFinder, and BestKeeper analyses disclosed that miR-221, let-7a, and miR-26a are the endogenous miRNAs most suitable for the normalization of serum exosomal miRNAs expression in liver carcinoma resection studies [60].

These tools have been widely applied to identify reference miRNAs also in whole plasma or serum samples. In serum samples from gastric cancer patients and healthy control, miR-16 and miR-93 resulted as the most stably expressed according to geNorm, NormFinder, BestKeeper algorithms, and comparative ΔCt method [61]. Moreover, they allowed to reveal that miR-101-3p and miR-93-5p are suitable for the normalization of miRNA expression in plasma of major depressive disorder patients and healthy individuals [62]. miR-93 was identified by geNorm and NormFinder software as an endogenous control also in a study that examined miRNA profiles in plasma samples from tuberculosis patients [63]. NormFinder, geNorm and DataAssist tools selected miR-92a-3p, miR-21-5p and miR-16-5p as internal normalizers for plasma miRNAs expression data in hypertensive patients [64].

Other normalization methods

In addition to normalization by endogenous controls, when the expression of many miRNAs is assessed, other methods can be used such as the global median or the quantile normalization or the cyclic loess that have been widely discussed in other works [65, 66]. miRNA profiling of serum, vitreous humor (VH), and exosomes of VH samples from uveal melanoma patients and healthy controls was performed by TaqMan low density array (TLDA). The global median normalization method, in which Ct values of each sample were normalized to the median Ct of the arrays, was used. The Pearson correlation between the Ct median and the Ct of each miRNA allowed to identify miR-320 and snRNA U6 as reference

miRNAs (with the expression profile close to the median of TLDAs) [67].

Spike-in (exogenous)

The addition of a known concentration of exogenous synthetic miRNA, before the RNA extraction step, is known as spike-in control. It allows to assess if observed variations in RNA expression are also due to the sources of variability like quality and efficiency of RNA isolation, cDNA synthesis, and PCR amplification. Synthetic RNA spike-ins should not be used for normalization, which should always be carried out with endogenous miRNAs. To this end, synthetic various non-human miRNAs are used, as for example, cel-miR-39 from *Caenorhabditis elegans* [13, 45] or ath-miR-159a from *Arabidopsis thaliana* [68].

However, the choice of using a spike-in, instead of real endogenous miRNAs [69–72], to normalize miRNA expression data should be accurately assessed. Also, when the mean and/or the median of the expression values of three *C. elegans* spiked-in miRNAs is used [73–76], it should be stated that a starting hypothesis is accepted and that the total RNA content, before extraction procedures, is the same in all samples.

Conclusions

In the last few years, increasing knowledge on exosome functions, mainly due to their functional content, has attracted great interest in the research of circulating miRNAs as biomarkers, especially regarding tumor diseases. Quantification of miRNA expression allows to appreciate the differential expression between healthy and patient groups. Although specific exosomal miRNA profiles are known for some diseases, there is not always consensus among different studies regarding the same kind of tumor. This could also be due to heterogeneous experimental designs that can lead to different results [70]. For example, both the extraction procedures and the operator represent variables that can alter the measures. For this reason, standard operating procedures must be applied at all levels (from sample collection to miRNA quantification) to make the assessment of miRNA expression reliable [32]. In particular, it is very important to perform the normalization of RT-qPCR data based on control miRNAs.

Unfortunately, there is no reference miRNA valid for every experimental design; therefore, it should be assessed in each experiment. Obviously, the best approach is to perform an assessment of many candidate controls, for example, by RNA array cards, including also the endogenous controls validated in other setups. In the absence of this possibility, controls validated in other setups could represent the starting point for validation in a specific experiment [54, 77].

Compliance with ethical standards

Conflicts of interest None

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