

Article

Paper-based Exosomal MicroRNA-21 Detection for Wound Monitoring: A Proof of Concept and Clinical Validation Trial Study

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Abstract: Emerging evidence has shown that microRNAs play pivotal roles in wound healing. MicroRNA-21 (miR-21) was previously found to upregulate in order to fulfill an anti-inflammation role for wounds. Exosomal miRNAs have been identified and explored as essential markers for diagnostic medicine. However, the role of exosomal miR-21 in wounds has yet to be well studied. In order to facilitate the early management of poorly healing wounds, we developed an easy-to-use, rapid, paper-based microfluidic-exosomal miR-21 extraction device to determine wound prognosis in a timely manner. We isolated and then quantitatively examined exosomal miR-21 in wound fluids from normal tissues, acute and chronic wounds. Eight improving wounds displayed lower levels of exosomal miR-21 expression after wound debridement. However, four instances of increased exosomal miR-21 expression levels were notably associated with patients with poor healing wounds despite aggressive wound debridement, indicating a predictive role of tissue exosomal miR-21 for wound outcome. Paper-based nucleic acid extraction device provides a rapid and user-friendly approach for evaluating exosomal miR-21 in wound fluids as a means of monitoring wounds. Our data suggest that tissue exosomal miR-21 is a reliable marker for determining current wound status.

Keywords: P-ELISA; exosome; microRNA-21; wound healing

1. Introduction

Early detection of wound status via wound biomarker examination has the potential to prevent acute and chronic wounds from worsening. In addition to facilitating early clinical detection of symptomatic wounds, wound-related biomarkers serve as surrogate markers to predict wound outcomes and promote prompt therapeutic intervention. Some wound-fluid or tissue-derived factors, such as metalloproteinase enzymes (MMPs), growth factors, and interleukins, have been reported to serve as potential markers for predicting wound outcomes [1].

It is clear that microRNA (miRNA), involved in gene regulation to control cellular pathways, has become a powerful diagnostic tool with medical implications. The role of miRNAs in skin wound healing is well-established [2]. MiRNA-155 was reported to be highly expressed in the inflammatory phase of wound repair [3]. MicroRNA-21 (MiR-21) has also been shown to have an anti-inflammatory role and was upregulated in macrophages when alleviating inflammation [4]. Extracellular vesicles such as exosomes have been developed as important mediators for transmitting intercellular biological signals, including miRNAs, to modulate cellular function [5]. Although nucleic acid testing (NAT) has been widely used for disease diagnosis, food safety control, and environmental

monitoring, it currently requires laborious off-chip exosome separation and nucleic acid extraction prior to detection. Exosomal miRNAs have been shown to act as better biomarkers than non-exosomal miRNAs for disease diagnosis [6]. However, using exosomal miR-21 to determine wound prognosis has not been studied until now.

Paper-based ELISA (P-ELISA), a well-developed and convenient tool in diagnostic medicine, has been widely applied to examine the state and severity of various diseases [7, 8]. We successfully identified high human neutrophil elastase expression levels in chronic wound fluids using P-ELISA [9]. Here, we developed an easy-to-use, rapid, paper-based microfluidic-extraction device to investigate wound status by analyzing exosomal miR-21 in wound fluids using real-time polymerase chain reaction (qPCR). Results may be used to help determine wound prognosis and consequently provide better wound management.

2. Results

2.1. Development of paper-based nucleic acid extraction device

To detect a particular nucleic acid in our wound fluid samples, we developed a 3D paper-based nucleic acid extraction device by coating Whatman grade 1 filter paper with silica to reinforce nucleic acid binding (Figure 1). Nucleic acids were then adsorbed onto the silica nanoparticle surface using a binding solution with a pH value lower than the pKa value of the silicon OH group (pH=4) [10]. In this manner, Whatman grade 1 filter paper was made suitable for use as a paper-based immunoaffinity device and a paper-based nucleic acid extraction device. Hydrophobic and hydrophilic areas were clearly divided by All Purpose Duct Tape DT8 (3M™) to create different microfluidic device types. Specific exosomal antibody, anti-CD63 antibody, was coated onto the device to isolate exosomes from wound fluids. Based on this design, sample fluid was added to the inlet port on the paper-based immunoaffinity device to capture exosomes by CD63 binding. After exosomal cleavage with lysis buffer, nucleic acids in the exosomes were adsorbed by the silica nanoparticle onto the paper-based nucleic acid extraction device in a salt buffer solution at pH higher than the pKa of silicon surface OH groups or in dd water at an elevated temperature (55°C). Morphology and size of captured exosomes were analyzed by SEM and qNano, respectively. We further used paper-based exosomal nucleic acid extraction devices and antibody conjugated to horseradish peroxidase to produce a colorimetric readout. The miRNA expression levels could be quantified by real-time polymerase chain reaction (qPCR) to assess wound status within 30 min.

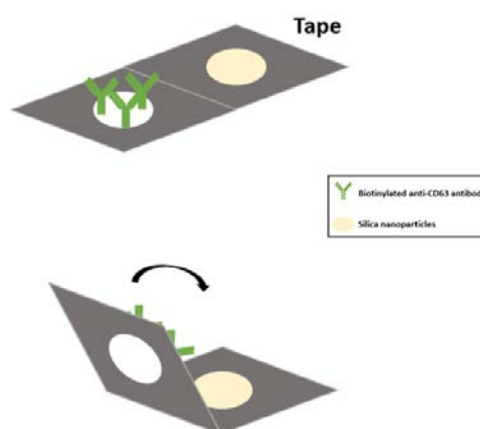


Figure 1. 3D paper-based nucleic acid extraction device to detect miR-21 in wound fluid.

To achieve rapid assessment of nucleic acid for point-of-care testing (POCT), a paper-based nucleic acid extraction device was developed to replace traditional nucleic acid extraction procedures. This approach requires no expensive or large instruments, and ultracentrifugation or ultrafiltration are not necessary. This paper-based approach easily handles exosome extraction and serves as an avenue for point-of-care testing development. In this process, Whatman grade 1 filter

paper was cut to a suitable size. The filter paper was coated with 20 μL of Silanol solution (3 mg/mL in dH_2O) on both sides and was desiccated at room temperature. In order to maintain clinical result consistency, all parameters such as adsorption time, elution time, temperature, and pH value used were fixed [10]. To improve the efficiency of the device and facilitate maximal adsorption and elution ability of nucleic acid, the binding buffer was set at $\text{pH}=4$. At room temperature, hydroxyl on the silanol surface reduced negative charge repulsion force and provided more room for hydrogen binding with nucleic acid. The adsorptive ability of nucleic acid was aided by a reduced electrostatic repulsive force and promoted by the hydrogen and possible salt bridge between nucleic acid and the silicon-surface OH group, which was relatively stable in the pH 4 and room-temperature environment. To meet POCT criteria, we aimed to assess nucleic acid levels at room temperature. According to a previous study, elution rate was better at 55°C than room temperature [11]. We then chose a pH 9 solution as our elution buffer and warmed it up to 55°C for 45 min to increase deprotonation and negative charges on the Silanol surface. Thus, elution of nucleic acid was made by cleavage of hydrogen bonds between silica and nucleic acid.

We then chose miR-21 samples to study elution time. Elution time points for observation via electrophoresis were 15 min, 30 min, 45 min, and 60 min (Figure 2A). The data show that elution concentration increased with time and reached its highest capacity at 45 min (Figure 2B). To reduce the unstable pH value and examination bias of qRT-PCR caused by the tiny sample volume, we selected ddH_2O as our binding and elution buffer. To determine optimal PCR reaction conditions, synthesized miR-21 was diluted into 109 copies/ μL , 108 copies/ μL , 107 copies/ μL , 106 copies/ μL , and 105 copies/ μL and applied to the paper-based nucleic acid extraction device. Samples were bound with ddH_2O for 3 min at room temperature and then desorbed for 45 min at 55°C . A PCR calibration curve and an amplification plot were used to determine the quantitative levels of miR-21.

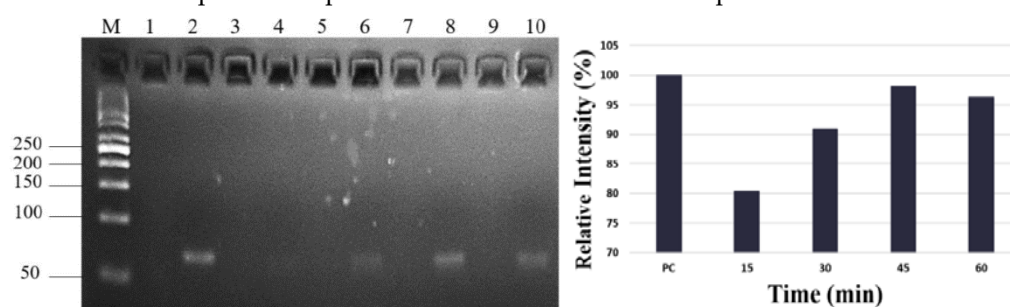


Figure 2. Elution time of miR-21 with electrophoresis (A, left) Elution times for observation of miR-21 were 15 min, 30 min, 45 min, and 60 min. (B, right) Quantitative data of miR-21 expression levels at different elution times. The data showed elution was increased with time and reached its highest capacity at 45 min.

2.2. The expression levels of exosomal miR-21 were higher than non-exosomal miR-21 from wound fluids

Previous study has shown that a majority of miRNAs are expressed in the exosomal serum of hepatocellular carcinoma patients [12] and exosomal saliva [13]. However, differences in miR-21 concentration in accordance with different wound conditions have not been previously addressed. We thus examined the differences between exosomal and non-exosomal miR-21 in wound fluids. Our data show that expression levels of exosomal miR-21 were higher than those for non-exosomal miR-21 from wound tissue (Figure 3).

To further understand the role of exosomal miR-21 in clinical wound status, we used a paper-based nucleic acid extraction device to investigate exosomal miR-21 in wound fluids. We first tested 3 chronic wound samples originating from three different body parts (left leg, right leg, left elbow) of one patient with multiple simultaneous injuries. Samples were added directly to the antibody-coated zones without preprocessing, and RT-qPCR was once again used as a finished product evaluation tool. The obtained Ct values show negligible differences in exosomal miR-21 levels between the three samples (Ct values, left leg= 32.86 ± 0.49 ; Right leg= 32.85 ± 0.40 ; left elbow= $33.67 \pm$

0.29), indicating that exosomal miRNA-21 in wound fluid was independent of the injured location as long as the same physiological and treatment conditions were experienced.

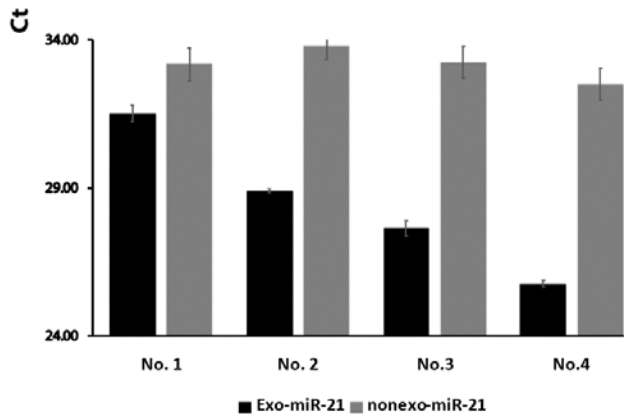


Figure 3. Comparison of expression levels of exosomal miR-21 and non-exosomal miR-21 in wound fluid. Exosomal miR-21 and non-exosomal miR-21 levels were investigated in 4 different wound samples. The data shows exosomal miR-21 expression level in wounds was higher than non-exosomal miR-21 expression level.

2.3. Higher exosomal miR-21 is associated with poor wound healing

A previous study demonstrated that all significant miRNAs could be detected in circulating exosomes of tongue cancer patients, compared to less detectable miRNAs in patient plasma [14]. We, therefore, studied the value of exosomal miR-21 and non-exosomal, free miR-21 in wound tissue for wound assessment. Exosomal miR-21 was isolated from patients with normal tissue and acute and chronic wound fluids. Normal tissue displayed a wide range of exosomal miR-21 expression. There were no significant differences in miR-21 expression between normal tissue, acute, and chronic wounds (Ct values, normal tissue=28.95+/-1.29, 25.77-31.69, n=11, acute=29.73+/-1.53, n=36; chronic=29.93+/-1.17, n=21, P=0.71). This result may be in keeping with a previous report indicating limitations in studying patient miRNAs due to such characteristics as age [15]. Although no significant differences in exosomal miR-21 expression were observed between acute and chronic wounds, we did observe a differential exosomal miR-21 expression in the same patient in accordance with different wound status. This study also examined changes in wound tissue exosomal miR-21 among 13 patients before and after wound debridement. Eight improving wounds displayed lower levels of exosomal miR-21 expression after wound debridement. However, 4 cases of increased exosomal miR-21 expression levels were noticed in poor healing wounds despite aggressive wound debridement (Table 1), indicating a predictive role of exosomal miR-21 for wound outcome (same patient).

Table 1. Expression levels of miR-21 before and after surgery.

Patient		miR-21 (Ct)		Wound type	Clinical outcomes
No.		Pre-surgery	Post-surgery		
1		29.31	30.11	chronic	improving
2		27.63	26.34	acute	worsening
3		30.3	27.2	chronic	worsening
4		32.2	28.91	chronic	worsening
5		26.84	26.33	acute	worsening

6	28.76	29.59	acute	improving
7	29.78	30.05	acute	improving
8	30.01	29.99	acute	Stationary
9	28.87	29.77	chronic	improving
10	30.72	31.53	chronic	improving
11	29.44	31.53	acute	improving
12	30.67	31.66	chronic	improving
13	30	31.48	acute	improving

3. Discussion

With the increasing need for point-of-care wound assessment, we developed an inexpensive and easy-to-use paper-based nucleic acid detection device to investigate exosomal miR-21 in wound fluids that was superior to traditional methods that require expensive infrastructure and time-consuming processes. This paper-based nucleic acid extraction device provides a potent, user-friendly approach for capturing exosomes for analysis by SEM and qNano to examine morphology and size, respectively. When exosomal miR-21 was successfully extracted from wound fluids, a colorimetric readout was produced within 30 min. The data was analyzed using real-time PCR to provide information for wound assessment. Our data suggest that exosomal miR-21 is a reliable marker for the early detection of wound status. The paper-based exosomal miR-21 extraction device developed in this study may be leveraged to produce a promising, first-of-its-kind screening tool for clinical wound monitoring.

MiR-21 is the most widely studied miRNAs in cancer and heart diseases [16, 17]. MiR-21 could be found in the cytosol, exosome, neoplastic and non-neoplastic cells [18]. As a result of interaction with cell membrane protein, exosomal miR-21 was less vulnerable to extracellular RNase degradation [16]. After activation, miR-21 could be released from exosome and remain stable by natural leakage in the serum or body fluids [19]. In addition, a previous study demonstrated a similar expression profile for miR-21 between tissue and plasma samples from breast cancer patients [20], and most studies have shown that a majority of miRNAs are expressed in the exosomal serum of hepatocellular carcinoma patients [12] and exosomal saliva [13]. Sensitivity was higher when using exosomal miR-21 as a biomarker for diagnosing hepatocellular carcinoma compared to detection by serum miR-21 [21], which further supports our finding that exosomal miR-21, rather than non-exosomal miR-21 in wound tissue, might be a better prognostic marker for wound assessment. Furthermore, miR-21 was detected in different kinds of body fluids such as urine, saliva, tears and breast milk [22]. MiRNAs in body fluids might indicate specific role associated with the surrounding tissues [22]. We showed here that exosomal miR-21 was exceptional stability present in the wound tissue fluids, indicating the development of exosomal miR-21 as a novel tool of tissue-based wound biomarkers.

MicroRNAs have recently been recognized as important regulators in skin wound healing [23]. They have been reported to regulate healing processes such as proliferation, migration, and angiogenesis [24]. MiR-21 was found to be up-regulated in activated epithelial cells and mesenchymal cells and involved in wound contracture and collagen deposition in mice [25]. However, differences in miR-21 concentration in accordance with different wound conditions have not been previously addressed. Our study highlighted the value of exosomal miR-21 in wound prognosis. A previous study demonstrated that higher miR-21 expression was related to the poor outcome of colorectal cancer patients [26]. Moreover, miR-21 was overexpressed in nonhealing venous ulcers [27]. Synthetic miR-21 was shown to inhibit epithelialization in a human skin organ culture wound model and to decrease granulation tissue formation in a rat model [27]. Consistent with that notion, our study suggests that higher exosomal miR-21 is associated with poor wound healing. Together, these results

demonstrate that paper-based devices are well suited for extracting tissue-derived exosomal miR-21 from wound fluids for the determination of wound status. Clinical validation of this device is, however, limited by the fact that low levels of wound exudate and complicating the wound environment may affect sample collection and the cross-section of exosomal miR-21 and substrate. Additionally, a temperature-controlled environment is a basic requirement for PCR amplification. Combining loop-mediated isothermal amplification with lateral flow assay or a field-effect transistor to examine exosomal miRNAs will allow our device to become more efficient and convenient and also increase the possibilities for clinical application. Additional investigations will be needed to confirm this hypothesis. Future study should be conducted regarding the sensitivity and specificity of exosomal miR-21 for detecting and monitoring various wound conditions. In conclusion, we establish proof-of-principle of paper-based extraction device for tissue-based exosomal miR-21 wound status detection by using clinical wound fluids from wound patients.

4. Materials and Methods

4.1. Patient samples

To study miR-21 expression in wound fluids, a total of 68 tissue samples (normal: 11, acute: 36, chronic: 21) were harvested using standard surgical treatment procedures. The normal tissue samples were obtained from patients undergoing reconstructive surgery. All samples were mixed with protein extraction buffer (RIPA, Merck, Millipore) and protein inhibitor (Merck, Millipore) and centrifuged at 4000 rpm for 30 min at 4°C. Informed consent was obtained from all patients receiving the treatment, and all study procedures were approved by the Institutional Review Board at National Cheng Kung University Hospital (No. B-ER-110-506).

4.2. Paper modification for exosome isolation

To isolate exosomes from multicomponent samples, we modified the surface of Whatman cellulose paper by adding anti-CD63 to specifically bind with tetraspanin protein CD63 on the exosomal membrane via specific and high-affinity antigen-antibody interaction. Briefly, circular pieces of Whatman cellulose chromatography paper (Grade 1) with a diameter of 6 mm were prepared as test zones and incubated in 50 μ L of 3-mercaptopropyl trimethoxysilane (MPTMS) solution (4% in 99% ethanol) and N- γ -maleimidobutyryloxy succinimide ester (GMBS) solution (0.01 μ mol/mL in 99% ethanol) for 30 minutes and 15 minutes, respectively. The papers were then incubated in 50 μ L of NeutrAvidin solution (10 μ g/mL in PBS) for 60 minutes at 4°C. Afterward, 20 μ L of 1% BSA (w/v) in PBS was added as a blocking solution 3 times every 10 min. Finally, 20 μ L of biotinylated anti-CD63 antibody solution (20 μ g/mL in PBS containing 1% (w/v) BSA) was dispensed onto the test zones 3 times every 10 min to bind with NeutrAvidin. Removing unbound molecules with three PBS washes provided us with anti-CD63-modified paper. Heterogeneous wound fluid samples (20 μ L) were dispensed onto these coated papers, which were incubated for 20 min to capture exosomes by virtue of the specific antibody-antigen binding. Unwanted materials were washed off with PBS. All steps were performed at room temperature unless otherwise stated.

4.3. Paper-based procedure for exosomal nucleic acid extraction

Once isolated, exosomes were lysed by adding 95°C ddH₂O to the experimental area, whereupon the released nucleic acids were adsorbed with silica particles. Specifically, 10 μ L of 95°C ddH₂O was slowly added to the exosome capture zones 6 times every 5 min, followed by the addition of 20 μ L of silica solution (3 mg/mL in diH₂O) to allow the silica particles to absorb the released nucleic acids. The experimental papers were then rinsed 3 times with 20 μ L of ddH₂O to remove unwanted materials before being immersed in a tube containing 20 μ L of ddH₂O (an equal volume to the originally used volume of sample) and incubated at 55°C for 45 minutes for nucleic acid elution. After incubation and paper removal, the solution obtained represented the exosomal nucleic acid solution with the same concentration as the original sample.

4.4. Paper-based procedure for free nucleic acid collection

After adding chronic wound fluid to the antibody-coated paper to capture exosomes and using PBS to wash away unbound molecules, the wash solution was collected to analyze it for free nucleic acids. A piece of paper with dimensions of 0.5 mm x 3 mm was prepared and silica particles were coated on the first third of the paper. The solution containing the obtained exosome-free nucleic acids was slowly added to the silica-coated paper. Leveraging the capillary phenomenon without the need for external force, the nucleic acids were absorbed onto the silica surface while other impurities followed the liquid flow to the rest of the paper. One-third of the paper containing nucleic acid-absorbing silica particles was cut off and dipped in 20 µL of ddH₂O (an equal volume to the originally used sample volume) and incubated at 55°C for 45 minutes for nucleic acid elution. After incubation and paper removal, the solution obtained represented the free nucleic acid solution with the same concentration as the original sample.

4.5. RT-qPCR for nucleic acid quantification

Reverse transcription and real-time PCR were used to analyze miRNA expression. These steps were performed using a Veriti™ Thermal Cycler and a StepOnePlus™ Real-Time PCR (RT-PCR) System (Applied Biosystems, USA), respectively. Experimental procedures and primer design were inspired as previously reported. (I. Balcells, S. Cirera, and P. K. J. B. b. Busk, "Specific and sensitive quantitative RT-PCR of miRNAs with DNA primers," vol. 11, no. 1, p. 70, 2011.). In the RT reaction, each 10.2 µL reaction mixture contained 1.0 µL of ATP, 1.0 µL of dNTP, 1.0 µL of 10X Poly(A) buffer, 1.0 µL of reverse transcription primer, 0.5 µL of reverse transcriptase, 0.2 µL of E.coli Poly(A) polymerase, 0.2 µL of RNase inhibitor, 4.3 µL of nuclease-free water, and 1.0 µL of RNA sample. The reverse transcription reaction temperature flow was 60 min at 42°C, 5 min at 95°C, and incubation was at 4°C. The reverse transcription product containing synthesized cDNA was then amplified using the SYBR Green qPCR method. The 10 µL of SYBR Green qPCR mixture included 5.0 µL of SYBR Green I Master mix, 1.0 µL forward primer, 1.0 µL reverse primer, 2.0 µL nuclease-free water, and 1.0 µL of reverse transcription product. Thermocycling conditions were 5 min at 95°C for initial heat activation, and 40 cycles of 10s at 95°C and 30s at 60°C for annealing. Finally, Ct values were measured to represent the expression of target miRNA.

Universal Reverse Transcription Primer: 5'-CAGGT CCAGT TTTT TTTT N-3

miR-21 Forward Primer: 5'-TCAGT AGCTT ATCAG ACTGA TG-3'

miR-21 Reverse Primer: 5'-CGTCC AGTTT TTTT TTTT TTCAA C-3'

4.6. Statistical analysis

Analysis of variance was used to determine statistical differences of exosomal miR-21 among normal tissue, acute, and chronic wound fluids. Values of $p < 0.05$ were considered statistically significant. The results were expressed as mean \pm SD.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Institutional Review Board at National Cheng Kung University Hospital (No. B-ER-110-506).

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