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ORIGINAL ARTICLE

First steps in the development of a liquid biopsy in situ hybridization protocol to determine circular RNA biomarkers in rat biofluids

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Abstract

Purpose Epigenetic factors are involved in the pathogenesis of congenital diaphragmatic hernia (CDH). Circular RNAs (circRNAs) are epigenetic regulators amenable to biomarker profiling. Here, we aimed to develop a liquid biopsy protocol to detect pathognomonic circRNA changes in biofluids.

Methods Our protocol is adapted from the existing BaseScope™ in situ hybridization technique. Rat biofluids were fixed in a gelatin-coated 96-well plate with formalin. Probes were designed to target circRNAs with significant fold change in nitrofen-induced CDH. FastRED fluorescence was assessed using a plate reader and confirmed with confocal microscopy. We tested maternal serum and amniotic fluid samples from control and nitrofen-treated rats.

Results We detected circRNAs in rat serum and amniotic fluid from control and CDH (nitrofen-treated) rats using fluorescent readout. CircRNA signal was observed in fixed biofluids as fluorescent punctate foci under confocal laser scanning microscopy. This was confirmed by comparison to BaseScope™ lung tissue sections. Signal was concentration dependent and DNase resistant.

Conclusion We successfully adapted BaseScope™ to detect circRNAs in rat biofluids: serum and amniotic fluid. We detected signal from probes targeted to circRNAs that are dysregulated in rat CDH. This work establishes the preliminary feasibility of circRNA detection in prenatal diagnostics.

Keywords Congenital diaphragmatic hernia · Circular RNA · Biomarker · Liquid biopsy · BaseScope™

Introduction

Congenital diaphragmatic hernia (CDH) is a developmental birth defect which complicates 2.3–2.8 per 10,000 live births [1–4]. It is among the most common congenital anomalies reported [3], and represents a significant public health issue and economic burden [5, 6]. Early diagnosis of CDH enables multidisciplinary antenatal counselling, delivery planning, and identification of cases suitable for in utero therapeutic intervention. CDH lacks a definitive, biomarker-based diagnostic and prognostic test. Accurate and scalable diagnostic tests are, therefore, imperative to identify CDH pregnancies in early gestation, and redirect goals of care as necessary.

The inability to identify a common genetic etiology in the majority of CDH cases [4, 7] has led to the hypothesis that epigenetic factors play a role. We have previously published that microRNAs are downregulated in abnormal lung development and CDH [8], and that prenatal microRNA therapy improves lung development in nitrofen-induced CDH and reduces the incidence of CDH [9]. Circular RNA (circRNA) species can regulate microRNAs and have thus emerged as candidate biomarkers. CircRNAs are covalently closed, non-polyadenylated, single-stranded RNA structures which act as powerful epigenetic regulators. They arise as a result of head-to-tail "back-splicing" events, in which the 5′ end of an upstream splice donor site is joined to the 3′ end of a downstream splice acceptor site. Various properties of circRNAs suggest that they are amenable to biomarker profiling: they are stable, conserved, enriched in biofluid samples,

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and expressed in a tissue- and development stage-specific pattern [10]. Tissue-specific changes in fetal RNA (including circRNA) expression are reflected in maternal blood during pregnancy [11]. Thus, circRNAs serve as viable and robust biomarkers for a prenatal "liquid biopsy".

Through microarray profiling (Arraystar Inc., USA) of human and rat tissues, our lab has identified altered circRNA expression in fetal CDH lungs relative to controls [Unpublished results]. In E21 rat lung tissue, two circRNAs of particular interest have emerged: rno_circRNA_007475 and mmu_circRNA_31436. These circRNAs have significant fold changes in nitrofen-induced CDH relative to controls, and have been validated using BaseScope™ in situ hybridization in FFPE lung tissue sections [Unpublished results]. Considering the known properties of circRNAs—that is, their stability, conservation between species, enrichment in biofluids, and spatiotemporally-specific expression—these microarray data suggest that altered circRNA expression profiles could be used as a diagnostic and/or prognostic aid in CDH. We hypothesize that altered circular RNA expression profiles can serve as prenatal biomarkers for CDH.

The current gold standard for detection and analysis of circRNAs is RT-qPCR, with outward-facing primers designed to hybridize to the back-splicing junction. For high-throughput analysis of samples, microarray profiling may be performed, with deep sequencing to verify results. Northern Blotting can also be used, but performs poorly given the relatively low abundance of circRNAs in the overall RNA population (necessitating large sample volumes). BaseScope™ is a proprietary in situ hybridization (ISH) technique developed by *Advanced Cell Diagnostics* for the visualization of short (50-300nt) RNA species with subcellular resolution. "*Z*"-shaped probes are designed in silico to hybridize in pairs to a specific target sequence. For analysis of circRNAs, these probes are designed with sequence complementarity to the back-splicing site. Adjacent hybridization of two "*Z*" probes to the target sequence forms a tail-binding site upon which an amplification tree is assembled. Chromogenic readout enables quantification of target expression with preservation of cellular architecture.

Multiple groups have successfully used BaseScope™ to profile circRNA species in FFPE tissue sections [12, 13]. To our knowledge, it has not yet been successfully applied to detect extracellular targets in biofluid samples. In this study, we sought to establish proof-of-concept data for the development of a circRNA-based liquid biopsy test for CDH. We aimed to adapt the existing BaseScope™ ISH assay and establish a new protocol to detect pathognomonic circular RNAs in rat biofluids using fluorescent readout. We verified this signal with direct visualization under confocal laser scanning microscopy.

Methods

Tissue harvesting for downstream applications

This study was approved by the health research ethics board from the University of Manitoba (19–010 (AC11436)). Animal studies were compliant with the ARRIVE guidelines [14]. Adult Sprague–Dawley rats were mated at night and dams considered as embryonic day 0 (E0) after confirmation of a sperm-positive vaginal smear. On E9, 100 mg nitrofen (suspended in 1 ml olive oil) was administered by oral gavage to a pregnant dam to induce CDH in pups. A control dam received 1 ml olive oil (without nitrofen) at the same gestational age.

Dams were euthanized at E21. Blood was collected from the maternal tail vein and serum was isolated by immediate centrifugation [15] and stored at − 80˚C. Fetuses were isolated and euthanized by cervical dislocation. Amniotic fluid was collected from individual sacs and stored at − 80˚C. Fetuses were dissected using microsurgery tools. The diaphragm was inspected caudocranially for the presence of the diaphragmatic defect. Left and right lungs were harvested and snap-frozen in liquid nitrogen or fixed in 10% formalin and embedded in paraffin.

Adaptation of BaseScope™ workflow for application to biofluid samples: experimental design and optimization

We developed a fixation protocol to immobilize liquid samples on an inert surface such that the BaseScope™ washing protocol can be tolerated without loss of sample. To limit prodigal use of valuable samples, preliminary fixation experiments were performed with human saliva. Saliva was supplemented with 10X formalin and added to a Superfrost™ Plus microscope slide. Slides were prepared with and without formalin, and compared to determine the effect on fixation. To purify RNA content, samples were treated with DNase using the Invitrogen™ TURBO DNA-free™ Kit (Thermo Fisher, AM1907). Slides were baked in a drying oven for 90 min at 37 °C. Baked slides were stained with Sybr Gold and DAPI, and washed in PBS for 2 min. Dried slides were coverslipped using EcoMount. Imaging was performed using epifluorescent microscopy (Zeiss, Germany) and images were processed using ImageJ software.

This protocol was sufficient to validate fluorescent signal from fixed biofluid samples. As BaseScope™ contains rigorous and multiple wash steps, nucleic acid loss is expected if performed on biofluid samples immobilized by conventional immunoabsorbant methods. To enable quantitative fluorescent readout, we next reformatted the existing BaseScope™ protocol for application to a 96-well microplate (Molecular

Probes®, Invitrogen™, M33089). To preserve the nucleic acid content, we immobilized biofluids onto a 96-well plate pre-coated with bovine gelatin (2% w/v) and dried at 40 °C. Biofluids were supplemented with 10× formalin and baked in a drying oven for 140 min (58 °C for 1 h, and 40 °C thereafter). Plates were subjected to the washing protocol for standard BaseScope™, using 1X RNAscope® Wash Buffer and performing incubations as per the BaseScope™ User Manual. Nucleic acid preservation was verified after successive washes using Sybr Gold staining.

BaseScope™ in situ hybridization on FFPE lung tissue sections

Probes were designed by *Advanced Cell Diagnostics* to hybridize specifically to two circRNAs with differential expression in the microarray experiment: rno_circRNA_007475 and mmu_circRNA_31436. Paraffin-embedded E21 rat lungs were sectioned and mounted on Superfrost™ Plus microscope slides (Fisher Scientific). Deparaffinization was performed using a standard xylene and ethanol series. The BaseScope™ assay was performed according to the BaseScope™ Detection Reagent Kit v2–RED User Manual, using the HybEZ™ II Oven. Tissue sections were examined using brightfield, epifluorescent, and confocal laser scanning microscopy at 20× and 40×. Images were processed using ZEN Imaging Software (Zeiss, Germany).

Application of modified BaseScope™ 96-well plate protocol to liquid samples

After optimizing the fixation procedure for biofluid samples, we proceeded to visualize target circRNAs in control adult rat serum immobilized on gelatin-coated 96-well plates. Serum samples were treated with Protease III and incubated at 40 °C for 10 min. Protease was not removed from the samples. BaseScope™ circRNA hybridization probe (rno_circRNA_007475, or mmu_circRNA_31436) and formalin (10×) were added to the protease-treated samples, which were vortexed, plated, and baked in a drying oven for 140 min (at 58 °C for 1 h, and 40 °C thereafter). Sample integrity was verified after successive washing stages using Sybr Gold staining to reflect total nucleic acid content.

Amplifiers from the BaseScope™ Reagent Kit v2-RED were diluted (1:10) with PBS. Diluted amplifiers were added to the wells in a stepwise fashion, following the incubation settings described in the BaseScope™ User Manual. The covered plate was placed directly into the HybEZ™ II Oven; the Humidity Control Tray was not used. Between incubation periods, all wells were washed twice with 100 µl RNAscope® Wash Buffer for 2 min. Wells were emptied by vacuum aspiration after each wash. FastRED detection reagent was prepared by combining RED-A and RED-B reagents in a 60:1 ratio, as per the BaseScope™ User Manual. This solution was diluted (1:10) with ddH_2O and 50 µl was added to each well, incubating for 10 min (covered). Wells were rinsed with 100 µl ddH_2O.

The plate was analyzed using the BMG Labtech FLUOstar® OPTIMA Plate Reader (Absorbance 544 nm, Emission 590 nm). Readouts were processed and statistical analyses were performed using RStudio. Additional verification of signal output was achieved with confocal laser scanning microscopy. Images were taken at 20× and 40× magnification and processed using ZEN Imaging Software (Zeiss, Germany).

Specificity of probe binding was confirmed by treating serum samples with DNase and repeating the above assay. NanoDrop™ spectrophotometry (Thermo Scientific) confirmed reduction in DNA content following DNase treatment.

Functional application of modified BaseScope™ protocol to detect circular RNAs in control and CDH biofluids

During the tissue harvest, amniotic fluid was collected from individual sacs and stored at − 80 °C. To establish whether circRNAs can be detected in amniotic fluid using our modified technique, our modified protocol was applied to amniotic fluid from control pups and nitrofen-induced CDH pups presenting with a diaphragmatic defect.

Statistical analysis

Statistical analysis was performed using RStudio [16, 17]. Dixon's Q test was used to exclude outliers. Where two independent groups were compared, Welch two-sample t test was used to assess significance. For more than two groups, statistical differences were analyzed using ANOVA, with Dunnett's post hoc analysis. P-values were adjusted with Benjamini & Hochberg correction [18]. Where significant, differences are denoted as $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$.

Results

Tissue harvesting for downstream applications

In the nitrofen-treated dam, six out of seventeen offspring were found to have a diaphragmatic defect (three large left-sided defects, and three small right-sided defects).

Experiment design and optimization

For saliva fixed to microscope slides, sample integrity was determined using Sybr Gold and DAPI staining to reflect nucleic acid content before and after washing. Sample fixation was improved with formalin supplementation (Fig. 1c). Unexpectedly, when samples were treated with DNase, widespread ~ 20 µm circular foci of intense DAPI staining appeared on the slides (Fig. 1e). We believe these represent contamination of the DNased sample with precipitate. Samples were supplemented with ethanol (2% of total volume) in an effort to improve DNA precipitation during DNase treatment. This successfully eliminated contaminants, but substantially reduced overall yield.

We redirected our assay to a 96-well plate format, using gelatin and formalin to enhance sample fixation, as described. Sybr Gold staining was again used to assess sample preservation with washing. With human saliva, this procedure achieved sufficient fixation to withstand the washing protocol for standard BaseScope™ (Fig. 1a).

BaseScope™ ISH for FFPE tissue sections

BaseScope™ was performed on E21 rat lung tissues using two circRNA target probes (rno_circRNA_007475 and mmu_circRNA_31436), and standard control probes. Slides were examined using brightfield, epifluorescent, and confocal microscopy at 20× and 40× magnification. We observed overlap between chromogenic and fluorescent signal output when images were overlaid (Fig. 2a–e).

Modified 96-well plate BaseScope™ protocol applied to control rat serum

After optimizing the biofluid fixation procedure using saliva, we proceeded to visualize the target circRNAs in control adult rat serum. Gelatin-coated plates and formalin supplementation (as described) preserved nucleic acid content (Sybr Gold fluorescence) with the stated washing protocol (Fig. 2h). Positive circRNA probe signal was observed in serum samples from control rats using a microplate reader. These findings were verified by visualizing punctate foci on confocal laser scanning microscopy (Fig. 2f, g).

We proceeded to optimize and validate the modified technique. We empirically deduced that 5–15 µl of control serum results in optimal fixation to maximize signal output for both probes, in a total volume of 75 µl per well (Fig. 3a). There was no significant change in fluorescence intensity for either circRNA probe (mmu_circRNA_31436 or rno_circRNA_007475) following DNase treatment of serum (Fig. 3b).

Modified 96-well plate BaseScope™ protocol applied to biofluids from control and nitrofen-induced CDH E21 rat fetuses

The modified 96-well plate BaseScope™ protocol was performed on serum and amniotic fluid samples collected from control and CDH pups at E21. Both hybridization probes (rno_circRNA_31436 and mmu_circRNA_007475) were tested. Signal output was not sufficiently high to produce a gross chromogenic change on the 96-well plate. We continue to optimize our protocol for serum and amniotic fluid fixation. Future research will establish optimal sample concentration to maximize signal output. However, punctate foci were observed when the plate was examined under confocal laser scanning microscopy at 20X (Fig. 3c–h).

Discussion

We have successfully adapted the BaseScope™ in situ hybridization protocol to produce an amplification signal in rat biofluids (serum, amniotic fluid). Our modified assay is an adaptation of the traditional BaseScope™ workflow, applied to a gelatin-coated 96-well plate. We detected signal from probes designed to hybridize to circRNAs that are dysregulated in nitrofen-induced CDH. We observed chromogenic and fluorescent signal output in serum and amniotic fluid samples from control and nitrofen-exposed rats. The signal output is concentration dependent and DNase resistant, and was verified with confocal microscopy. To our knowledge, this represents the first successful application of the BaseScope™ workflow to biofluid samples.

Our modified biofluid BaseScope™ assay has notable implications for circRNA detection techniques and the concept of the antenatal liquid biopsy. With pre-coated 96-well plates, our entire protocol can be completed and output analysed within 10 h, with limited hands-on time. Extensive tissue processing is not necessary, and concurrent fixation and hybridization further reduce the duration of the assay.

Existing circRNA detection techniques are laborious, time-consuming and error-prone. BaseScope™ offers significant advantages over RT-qPCR and Northern Blotting in terms of specificity, sensitivity, and benchwork. BaseScope™ circumvents the need for PCR primer design and validation, and an RNase-free environment is not required to successfully complete the assay. It combines the specificity and sensitivity of RT-qPCR with the morphological context of traditional ISH, delivering rapid results with a user-friendly protocol. The stringent in silico probe design algorithm used by ACD ensures probe binding specificity to maximize target hybridization with minimal off-target binding. To date, the limitation of BaseScope™ has been its inapplicability to liquid samples. We have overcome this

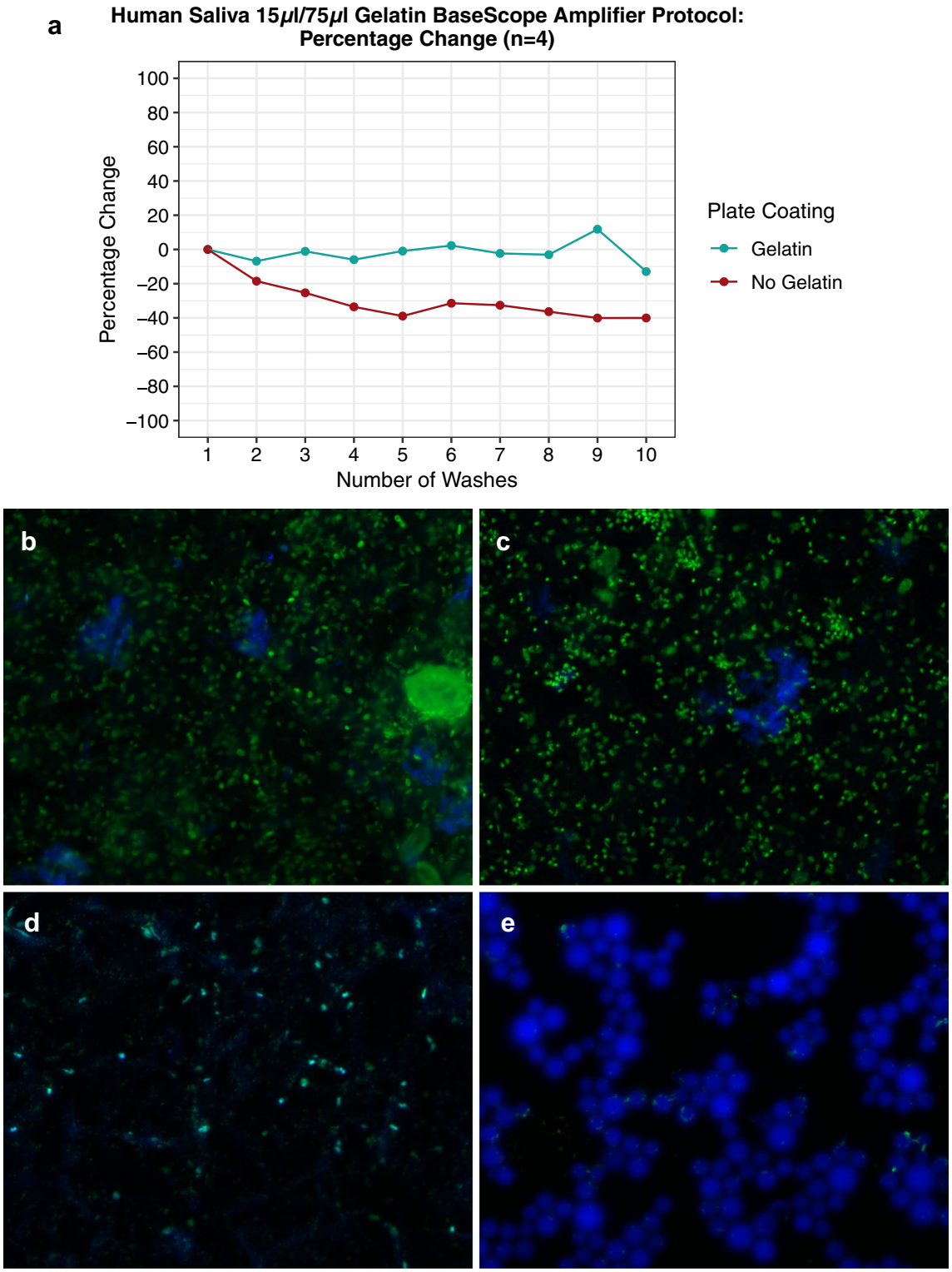
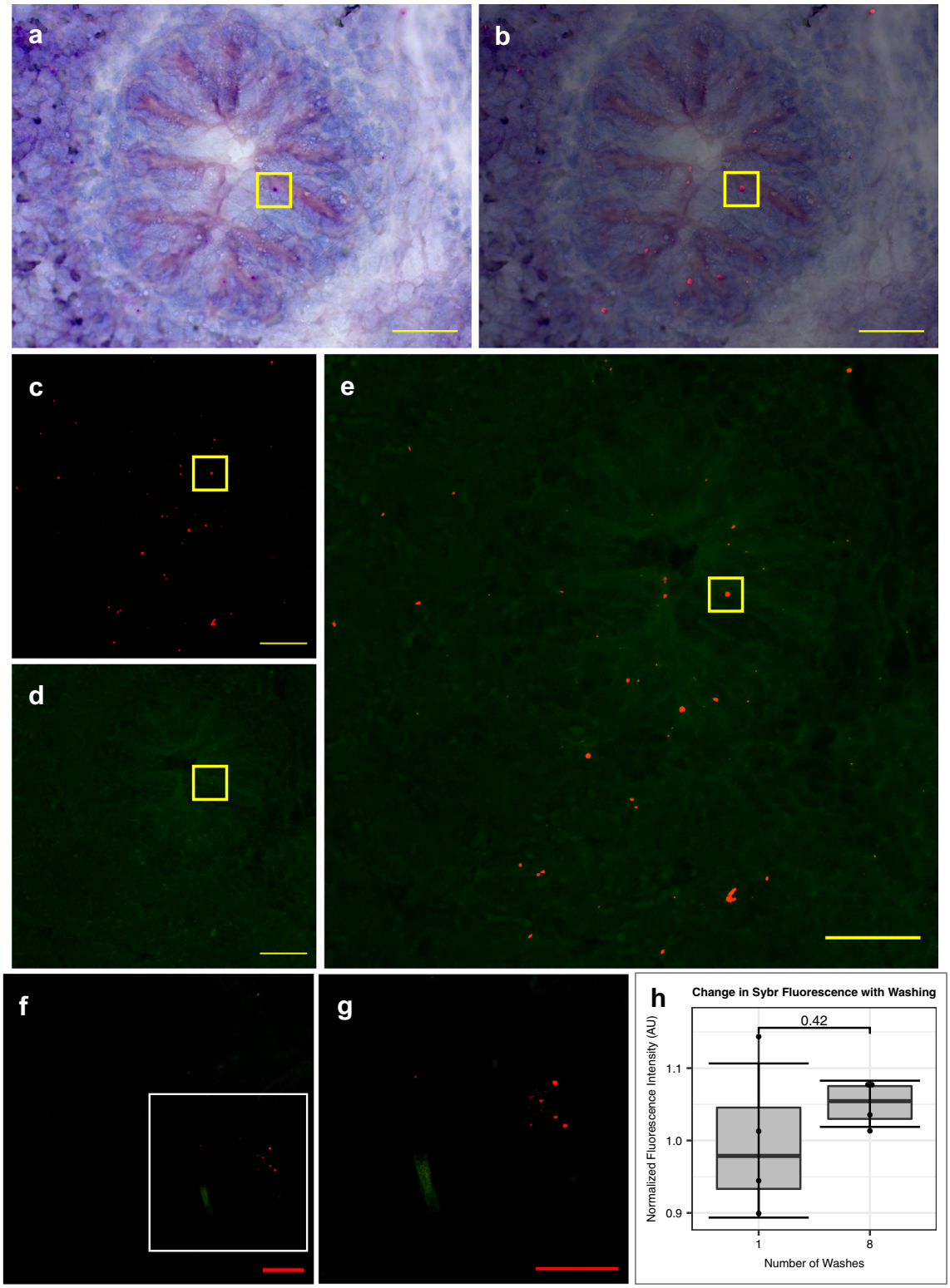


Fig. 1 Human saliva was fixed onto microscope slides using formalin and baking. Slides were stained with Sybr Gold (green) and DAPI (blue, and examined at 20× magnification. **a** Maintenance of sample integrity (Sybr Gold fluorescence) for human saliva fixed to a 96-well plate throughout the BaseScope™ wash protocol. **b** Without formalin. **c** Saliva supplemented with 10X formalin. **d** Slide washed with PBS. **e** Saliva sample treated with DNase and washed with PBS


a
b
c
d
e
f
g
h
Change in Sybr Fluorescence with Washing
0.42
Normalized Fluorescence Intensity (AU)
1.1
1.0
0.9
1
8
Number of Washes

◂**Fig. 2** Sample integrity is maintained with fixation to allow visualisation of FastRED signal in control rat serum using laser scanning microscopy. BaseScope™ in situ hybridization was performed on E21 control rat lung tissue sections for rno_circRNA_007475. **a** BaseScope™ FastRED fluorescent signal appears as punctate foci in airway epithelium under standard brightfield microscopy. Fluorescent signal demonstrated in (**c**) overlaps with chromogenic signal in (**a**) when images are overlaid, image (**b**). **d** Autofluorescence shows cellular architecture. **e** Signal is visualized relative to airway structure using confocal microscopy. A yellow box identifies a single circRNA signal focus within images (**a**) through (**e**). The modified BaseScope™ protocol was performed using control rat serum in a gelatin-coated 96-well plate. Signal output was determined by FastRED fluorescence, and was visualized using confocal microscopy at 20× and 40× magnification. Punctate signals are noted in (**f**) (enlarged in (**g**)). Autofluorescent gelatin aggregates are also observed. Red: FastRED; green: autofluorescence. Scale bar = 20 µm in images (**a**–**e**), 50 µm in images (**f**) and (**g**). **h** E21 control rat serum was added to a gelatin-coated 96-well plate and baked. Wash cycles were performed by adding RNAscope® Wash Buffer to wells and aspirating. Total nucleic acid content (indicated by Sybr Gold fluorescence intensity) was determined before and after the BaseScope™ washing protocol

issue by reformatting the assay, and have gathered proof-of-concept preliminary data to support the feasibility of this new protocol in a clinical setting.

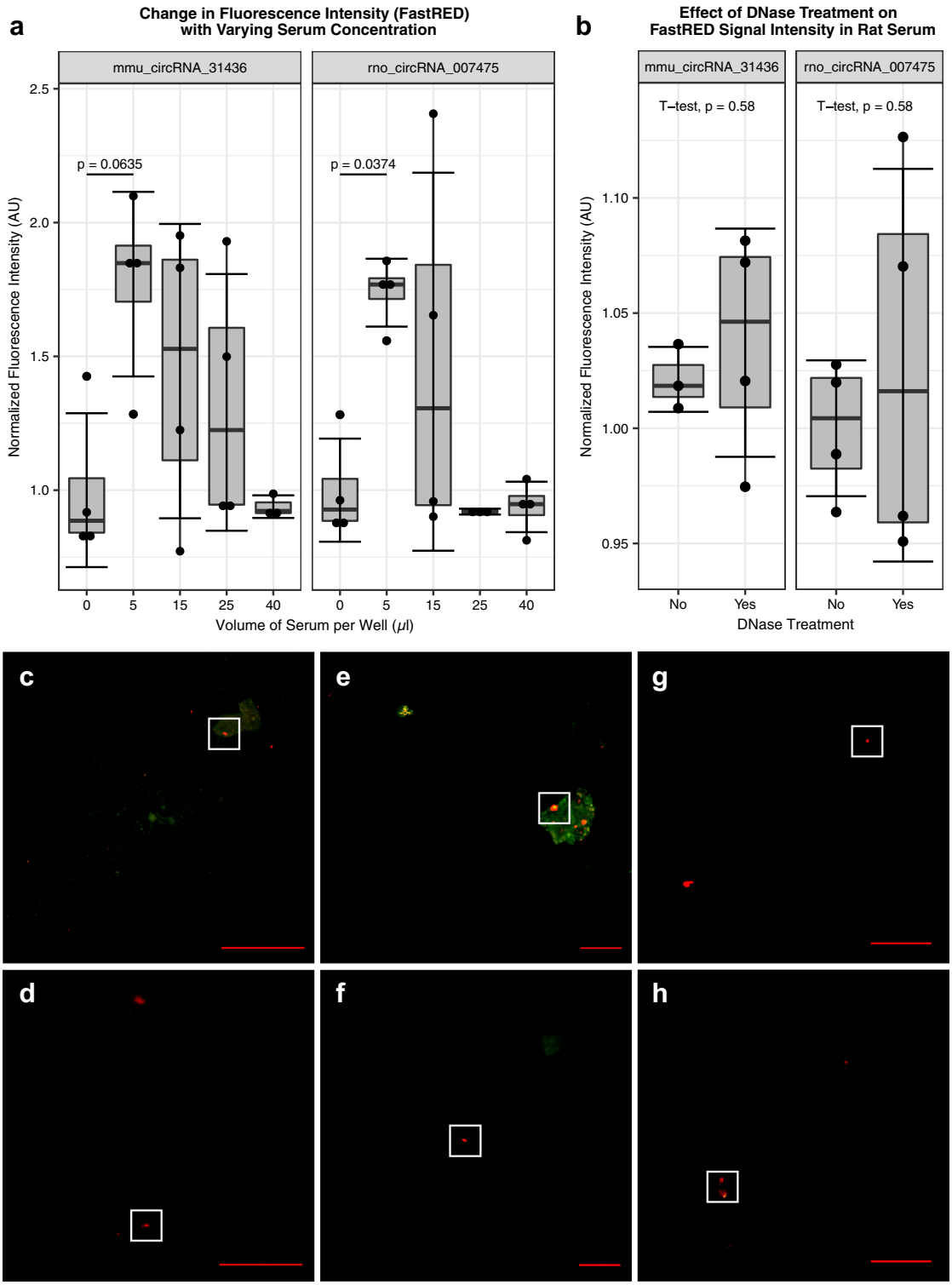
There is an increasing clinical need for a prenatal diagnostic and prognostic test for CDH. Ultrasound remains the mainstay of prenatal CDH diagnosis, but lacks sensitivity and prognostic insight into early gestation. Routine prenatal ultrasound identifies less than two-thirds of CDH pregnancies [19, 20], with a mean gestational age above 25 weeks [7]. This is problematic, as fetoscopic endoluminal tracheal occlusion (FETO)—currently the most promising prenatal therapy—is typically performed between 27–29 weeks of gestation [21]. Unlike a biomarker-based laboratory test, radiographic diagnosis is limited by geographic access to an institution equipped to deal with CDH. Even with successful prenatal diagnosis, prognostic insight is limited with existing radiological techniques. Ultrasound-based metrics—such as the ratio of observed-to-expected lung area to head circumference (O/E LHR)—perform poorly in early gestation [22] and are confounded by laterality [23]. Prenatal MRI and echocardiography offer potential added prognostic value [24, 25], but are generally only performed after diagnosis is established.

Although discovered over 20 years ago [26, 27], the prevalence and relevance of circRNAs has only come to the fore within the last number of years. The diagnostic and prognostic significance of altered circRNAs in CDH indicates that they are suitable candidates for liquid biopsies. We have established that two dysregulated circRNAs can be detected using our modified BaseScope™ technique in rat serum and amniotic fluid. The clinical translatability of these findings is significant. Amniocentesis is performed in the majority of pregnancies with suspected CDH, and routine bloodwork is performed in the clinical setting for expectant mothers. (Figure 4) presents a summary of the proposed liquid biopsy workflow based on these findings.

Future directions for this research involve further optimization of the liquid biopsy 96-well plate BaseScope™ technique. One major limitation of the current experiment is the absence of a robust positive control. The standard BaseScope™ positive control probes target mRNA from the intracellular housekeeping gene *PPIB*. This is not appropriate as a direct comparison to probes targeting circRNAs. In the absence of an appropriate positive control, it has not been possible to develop a standard curve or proceed with quantitative analysis of circRNA expression in our samples. This must be treated as a priority for this research to succeed. With a robust positive control, we can proceed to determine whether the circRNA fold changes observed in lung tissue are also reflected in biofluids.

In search of a positive control species, it may be prudent to review the E21 rat lung microarray data and identify a circRNA with unchanged expression in control and nitrofen-treated animals. This would have to be individually established for each biofluid sample, with a baseline expression that is sufficient to be detected by our assay. Another possibility involves "spike-in" of a bacterial circRNA species into the sample, with probes designed to target the bacterial circRNA. This is an area of future investigation.

Although this protocol produces detectable signal in its current format, it is possible that the assay we describe is not sufficiently powered to detect the expressed fold changes for the circRNAs of interest. If this is the case, it may be necessary to enrich or purify the circRNA content of the sample. In this regard, knowledge of the functional properties of circRNAs can be exploited: circRNAs are enriched in extracellular vesicles, including exosomes [28]. Cieslik and colleagues have described and validated a protocol for circRNA profiling using an exosome capture transcriptome protocol [29]. CD9 antigen is a tetraspanin glycoprotein expressed on cell and exosome membranes. As a future directive, coating 96-well plates with an anti-CD9 capture antibody may enhance exosome fixation and immobilization. Protease treatment of samples (which is included in the current protocol) will enable permeabilization to visualize circRNAs present in exosomal compartments.


a
Change in Fluorescence Intensity (FastRED)
with Varying Serum Concentration
mmu_circRNA_31436
rno_circRNA_007475
p = 0.0635
p = 0.0374
Normalized Fluorescence Intensity (AU)
2.5
2.0
1.5
1.0
0
5
15
25
40
Volume of Serum per Well (μl)
b
Effect of DNase Treatment on
FastRED Signal Intensity in Rat Serum
mmu_circRNA_31436
rno_circRNA_007475
T–test, p = 0.58
T–test, p = 0.58
Normalized Fluorescence Intensity (AU)
1.10
1.05
1.00
0.95
No
Yes
DNase Treatment
c
e
g
d
f
h

◂**Fig. 3** **a** Control adult rat serum was added to a gelatin-coated 96-well plate at varying concentrations, in a total volume of 75 µl per well. BaseScope™ probes were added and the plate was baked. BaseScope™ amplifiers were added sequentially and incubated, washing between incubations. FastRED fluorescence was determined using a plate reader. **b** Control adult rat serum was treated with DNase I. The modified 96-well plate BaseScope™ assay was performed using DNase-treated and DNase-untreated samples, for both circRNA target probes. Signal output was determined by FastRED fluorescence using a plate reader. The modified BaseScope™ protocol was performed on rat amniotic fluid collected at E21. Signal output was determined by FastRED fluorescence. This was visualized using confocal laser scanning microscopy at 20X magnification. With the probe targeting rno_circRNA_007475, signal was observed in control serum (**c**), nitrofen-exposed maternal serum (**d**), control amniotic fluid (**e**) and CDH (nitrofen-exposed) amniotic fluid (**f**). With the probe targeting mmu_circRNA_31436, signal was observed in control amniotic fluid (**g**) and CDH (nitrofen-exposed) amniotic fluid (**h**). Red: FastRED; green: autofluorescence. Scale bar = 50 µm

We provide convincing preliminary evidence to support the feasibility of a circRNA-based liquid biopsy for CDH. We have established that circRNAs are expressed in biofluids and have developed a novel protocol for their detection. Evidently, this project remains in its early stages, with a significant degree of validation and optimization required. However, our findings have potentially broad implications and merit further investigation. Ultimately, subject to validation and optimization, this protocol could in theory be applied to quantify circRNAs in human biofluids. This would represent a biofluid-based diagnostic test—or liquid biopsy—for CDH and other congenital anomalies.

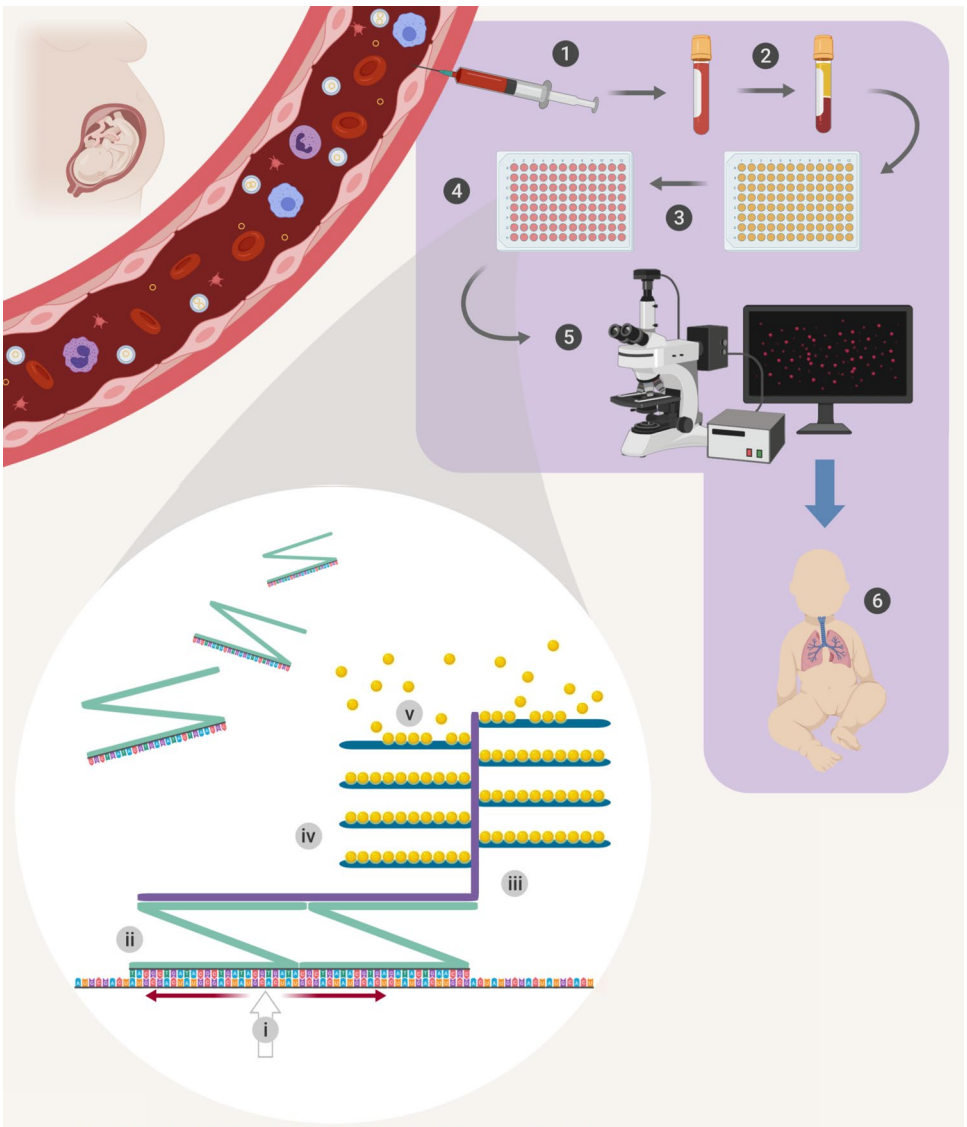


Fig. 4 (1) In the clinical setting, at prenatal workup, a routine blood sample is collected by venepuncture. (2) Serum is isolated by centrifugation. (3) The modified 96-well plate BaseScope™ assay is performed using specific circRNA probes. (4) FastRED detection reagent produces chromogenic and fluorescent amplification signal. Fluorescence is measured and compared to a known standard, enabling diagnosis / prognostication. (5) Signal verification can be obtained by visualization on confocal microscopy. (i) Probes are engineered with sequence complementarity to the circRNA back-splice junction. (ii) Probes are incubated with sample and hybridize to target circRNA sequence. (iii) Preamplifier (purple) is added and hybridizes to the binding site created by adjacent "double-Z" probes. (iv) A series of amplifier probes (blue) are added and incubated. These bind to the preamplifier to build an "amplification tree". (v) Label probe (yellow) is added and binds to amplifier branches. This produces fluorescent or chromogenic signal output

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants performed by any of the authors. All animals were sourced from the University of Manitoba Bannatyne Campus Animal Care and Veterinary Services facility. All procedures performed in studies involving animals were in accordance with the health research ethics board from the University of Manitoba (19–010 (AC11436)). Animal studies were compliant with the ARRIVE guidelines [14].

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