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14. ABSTRACT

This project explores the potential for small fragments of genetic material called transfer RNA-derived fragments (tRF)s as novel diagnostic, early disease and disease progression biomarkers as well as targets for therapy in malignant mesothelioma. In the first two years of the study most of the research effort was in sample preparation and PCR analysis of the two patient sample cohorts of over 1000 samples. Data emanating from the analysis of the first patient cohort of 400 samples did not support the original observations presented in the preliminary data cohort. Due to identified potential technical issues related to the way the samples were initially run, all samples were reanalysed. We are currently awaiting statistical analysis data. The second cohort of 600 samples (including 100 new controls) were also prepared and analyzed and we are awaiting statistical analysis data. We also continued to examine functional roles of the tRFs in mesothelioma cells and their effect on tumor growth, but these studies have been limited due to difficulty getting reagents from suppliers. Furthermore, reagents initially used to inhibit tRF cellular expression did not successfully block the tRFs and so have had to be redesigned to improve effectiveness. The progress of this study has been severely impacted by the death of CI Prof William Musk, who was integral in the statistical analysis of data. His death, together with the retirement of Prof Nick deKlerk has significantly delayed our ability to perform statistical analysis of the data. This, together with further waves of COVID has delayed the progress of the study and as such we have applied for and been granted a 12 month extension to the study.

15. SUBJECT TERMS

transfer RNA-derived fragments (tRFs), biomarker, malignant mesothelioma

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1. Introduction

This study examines specific small non-coding RNA fragments called transfer RNA-derived fragments tRF^{Lysa}, tRF^{LysB}, tRF^{ThrA} and tRF^{LeuA} as potential biomarkers for diagnosis, early screening and tumor progression of malignant mesothelioma and will determine if they have significant biological relevance for mesothelioma growth, which would make them novel molecular targets for therapy. In the short term this study aims to identify a tRF signature that will be used for screening high risk populations, providing earlier and more accurate diagnosis and monitoring disease progression, reducing the need for invasive biopsies and reducing stress to patients. In the long term, understanding the role of tRFs in mesothelioma growth may allow us to identify novel targets that will be used to develop better treatments to improve patient survival.

2. Keywords

Malignant mesothelioma, transfer RNA-derived fragments (tRF), biomarker, diagnosis, disease target, cell signalling, antisense oligonucleotides, mimics.

3. Accomplishments

The percentage completed of each of the task is outlined below. We have changed the order of some tasks due to issues related to COVID.

Specific Aim 1. Determine if tRF^{LysA}, tRF^{LysB}, tRF^{ThrA} and tRF^{LeuA} are potential diagnostic, early disease and disease progression biomarkers for malignant mesothelioma.

Major Task 1. Investigate tRF^{LysA}, tRF^{LysB}, tRF^{ThrA} and tRF^{LeuA} as diagnostic markers for malignant mesothelioma.

Subtask 1. Using real time PCR, measure expression levels of tRF^{LysA}, tRF^{LysB}, tRF^{ThrA} and tRF^{LeuA} in mesothelioma and control samples (Australian cohort of 400 samples).

- HRPO approval obtained.
- Improved methods are available to harvest and measure small non-coding RNA. We examined two different isolation approaches and compared tRF levels in control serum samples to determine the best approach. We demonstrated that the best approach was the original approach that we used in the preliminary studies and so all isolation and expression studies were performed using this approach (Appendix 1).
- Cohort 1 (400 Australian samples) prepared and tRF levels measured.

Timeline proposed months: 1-4. Timeline actual months: 1-10 (completed).

Subtask 2. Perform analysis on data to identify diagnostic signature

- Preliminary data analysis completed. Initial data does not support the preliminary data (Appendix 2), however, a more thorough analysis is currently being performed by Prof Nick de Klerk.

Timeline proposed months: 5. Timeline actual months: 50% completed.

Subtask 3. Obtain frozen serum samples from NYU and measure expression levels of tRF^{LysA}, tRF^{LysB}, tRF^{ThrA} and tRF^{LeuA} in mesothelioma and control samples to validate results from the Australian patient cohort.

- NYU patient serum samples have been shipped and processing completed

Timeline proposed in months: 6-8. Timeline actual in months: 18 (completed).

Subtask 4. Compare tRF^{LysA}, tRF^{LysB}, tRF^{ThrA} and tRF^{LeuA} expression data in NYU samples with Australian cohort to identify patients with mesothelioma compared with controls. Analysis will be done blind. Prof Nick de Klerk will analyse data once the reference panel has been established from subtask 2.
Timeline proposed in months: 10. Timeline actual in months: 0% completed.

Major Task 2. Investigate tRF^{LysA}, tRF^{LysB}, tRF^{ThrA} and tRF^{LeuA} as early disease markers for MM.

Subtask 1. Using real time PCR, measure expression levels of tRF^{Lysa}, tRF^{LysB}, tRF^{ThrA} and tRF^{LeuA} in mesothelioma and control samples collected every year for 10 years prior to being diagnosed with mesothelioma.

- Patient serum samples have been identified for the time course study (500 samples).
- We evaluated the effect of sample age on expression of miRNA in human serum samples to confirm that we can detect tRFs up to 10 years old (miRNA was measured while waiting for tRF primers). miRNAs were detected at constant levels up to 10 years after collection, confirming the feasibility of the proposed time-line study (Appendix 3.).
- This task will only be performed if data from major task 1 supports tRF^{Lysa}, tRF^{LysB}, tRF^{ThrA} or tRF^{LeuA} as a biomarker for mesothelioma.

Timeline proposed in months: 9-11. Timeline actual in months: 15% completed.

Major Task 3. Investigate tRF^{Lysa}, tRF^{LysB}, tRF^{ThrA} and tRF^{LeuA} as markers of disease progression for malignant mesothelioma.

Subtask 1. Using real time PCR, measure expression levels of tRF^{Lysa}, tRF^{LysB}, tRF^{ThrA} and tRF^{LeuA} in mesothelioma and control samples collected at time of diagnosis and compare with samples collected following treatment or palliative care for up to 24 months.

- This task will only be performed if data from major task 1 supports tRF^{Lysa}, tRF^{LysB}, tRF^{ThrA} or tRF^{LeuA} as a biomarker for mesothelioma.

Timeline proposed in months: 12-14. Timeline actual in months: 0% completed.

Specific Aim 2. Determine the biological significance of tRF^{LysA}, tRF^{LysB}, tRF^{ThrA} and tRF^{LeuA} in malignant mesothelioma cells and tumors.

Major Task 1. Determine the biological role of tRF^{LysA}, tRF^{LysB}, tRF^{ThrA} and tRF^{LeuA} in malignant mesothelioma cells.

Subtask 1. Identify the best cell lines to examine differentially expressed tRFs.

- Mesothelioma cell lines have been assessed for tRF levels to determine the best lines to use for functional studies. Mouse tRF primers are not commercially available therefore several designed primers are being assessed. Preliminary results show good expression of all tRFs in human cell lines but expression levels are low in mouse cell lines (Appendix 4). We assessed a number of other mouse tRF primers but expression levels were also low in the mouse cell lines examined.

Timeline proposed months: 8-9. Timeline actual months: 16 (completed).

Subtask 2. Determine functional roles of tRFs in mesothelioma cell lines by knocking down or overexpressing tRFs.

- Initial antisense oligonucleotides designed to inhibit tRF function did not significantly affect tRF levels in mesothelioma cells in vitro. New antisense oligonucleotides were designed and are currently being assessed.

Timeline proposed months: 10-18. Timeline actual months: 10% completed.

Subtask 3. Determine effect of knocking down or overexpressing tRFs in animal models of malignant mesothelioma.

- Tumors have been grown in mice for all 6 mouse tumor cell lines. tRF expression in each tumor is being assessed to establish baseline levels.

Timeline proposed months: 16-24. Timeline actual months: 15% completed.

Training and professional development
Nothing to report.

Dissemination of results
Nothing to report.

Plans for the next 12 months
The timelines of this study have been significantly affected by COVID, the retirement of PI Nick de Klerk and the death of PI Professor William Musk. Because of these events, we requested and were granted a 12 month project extension. Although most aspects of the study will remain unchanged, if we conclude that our data does not support tRFs as a biomarker for mesothelioma, we will not continue with Aim 1, major tasks 2 and 3 of the study. Aim 2 will continue as planned.

4. Impact

Impact on the development of the principal discipline(s)
Nothing to report.

Impact on other discipline(s)
Nothing to report.

Impact on technology transfer
Nothing to report.

Impact on society beyond science and technology
Nothing to report.

5. Changes/Problems

Changes in approach and reason for change
The overall approach to the study has not changed but we included more optimization studies and changed the order of some of the tasks. Optimization studies were performed as new improved kits for extraction of small non-coding RNA were available commercially. However, we demonstrated that the original method planned was still the most appropriate. We also changed the order of some of our tasks due to COVID delays). In addition, we had to repeat several studies as the original results differed from expected findings. If we conclude that our data does not support tRFs as a biomarker for mesothelioma, we will not continue with Aim 1, major tasks 2 and 3 of the study.

Actual or anticipated problems or delays and actions or plans to resolve them
COVID has been a major disruption throughout this study. In Australia we have had tight lockdowns which restricted our time in the laboratory, access to equipment and ability to obtain commercial reagents. In addition, we underestimated how long it would take to process and measure tRFs in the serum samples. All measurement have now been completed but with the retirement of PI Nick de Klerk and death of PI William Musk, statistical analysis of our data has been significantly delayed. Due to a shortage of appropriate statistical expertise available, we have had difficulty finding an appropriate person to analyse the data. PI Nick de Klerk has promised to assess the data in the very near future, so we hope this issue has been resolved. Animal studies have also been delayed until we find antisense oligonucleotides that affect tRF cellular expression. With the help of Thermo Scientific, we have now designed a new range of antisense oligonucleotides that will be assessed before Jan 2023.

Changes that had a significant impact on expenditures
The cost of the study has significantly increased because of 1. COVID, 2. A significant increase in inflation and 3. a major change in the exchange rate of the Australian compared with US dollar. These issues have effectively increased our consumable costs by approximately 30%. Most of these extra costs have been provided through internal Institute for Respiratory Health funds. However, as the salary for the Research Assistant on this study has been expended, PI Mutsaers will be performing the rest of the studies on his own.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
No change.

6. Products

Nothing to report.

7. Participants & Other Collaborating Organizations

What individuals worked on the project?

Steven Mutsaers, no change.
Cecilia Prele, no change.
Nick de Klerk, retired November 2021 but will still complete the study as planned.
William Musk, Deceased early 2022.
Marisa Ryan (Senior Research Assistant). Position terminated March 2022 due to insufficient funds.
Harvey Pass, no change.

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to report.

What other organizations were involved as partners?

Nothing to report.

8. Special Reporting Requirements

N/A.

9. Appendices

1. Optimization of tRF amplification in serum samples

We assessed two methods to determine the best approach to amplify the four tRFs (tRF^{LysA}, tRF^{LysB}, tRF^{LeuA} and tRF^{ThrA}) extracted from serum samples. In preliminary studies we used a modified method using the Invitrogen mirVana PARIS Kit (utilizing Phenol-Chloroform RNA extraction method) and QIAGEN RNeasy Mini Spin Columns. We wanted to compare this time-consuming and costly process with the automated QIAGEN miRNeasy Serum/Plasma Kit, to determine if this method could achieve similar or better RNA quality and reduce processing time.

Frozen serum samples from healthy controls, mesothelioma patients and lung cancer were processed using the two different RNA extraction methods. Real-time PCR CT values of all four tRFs and two housekeeping genes (miR16 and U6snRNA) demonstrated differences between the two kits for all serum volumes used (50, 100 and 200 µl), with lower CT values (i.e. higher levels of tRFs) using the mirVana protocol.

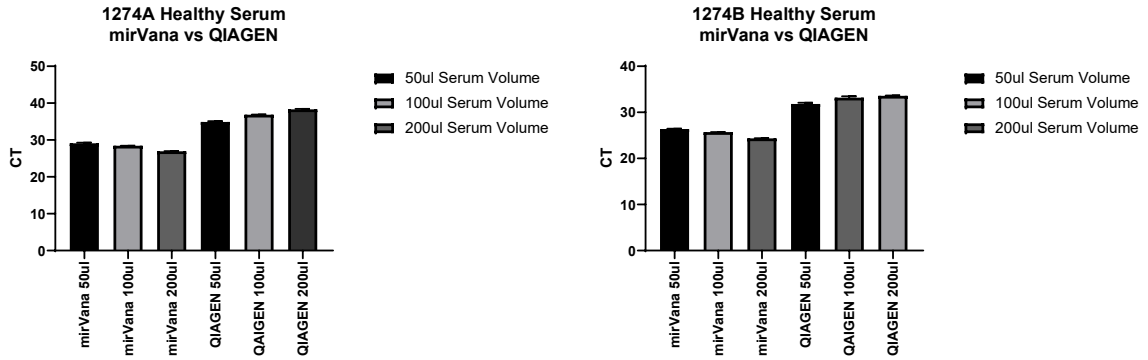

Figure 1. mirVana miRNA extraction kits give higher yields of tRFs than QIAGEN kits.
Levels of tRF^{LysA} (1274A) and tRF^{LysB} (1274B) extracted from different volumes of human serum using miVana and QIAGEN miRNA extraction kits. Lower CT values correspond to higher levels of tRFs (n=3).

The 200 µl serum volume had the lowest CT value in all six TaqMan assays (data not shown). Therefore, the modified mirVana protocol was used to process all serum samples, with an initial extraction volume of 200 µl of serum. As the modified mirVana protocol was costly due to the addition of the QIAGEN RNeasy mini spin columns, we examined various washing volumes in the hopes of increasing the number of extractions that could be performed per kit. The normal washing protocol (700 µl of wash buffer 1 and 2 x 500 µl of wash buffer 2/3) was compared to two reduced washing steps (A: 350 µl wash buffer 1 plus 2 x 250 µl wash buffer 2/3; B: 500 µl wash buffer 1 plus 1 x 500 µl wash buffer 2/3). There was a clear difference in the ΔCT values of the four tRF assays (data not shown), so we continued on using the normal volume of washing steps.
We also assessed the two housekeeping genes, miR16 and U6snRNA to confirm their suitability to normalise the tRF data. There is a large amount of literature regarding the "ideal" housekeeping genes to be used for different samples and different targets, but there was no recommendation for the tRFs we are measuring. Comparing the ΔCT values for mir16 only, U6snRNA only and the average of both, there was obvious differences between the two RNA extraction methods in regards to the U6snRNA only data and the data obtained by using the Average of the two housekeeping genes, compared to miR16 only data. This may be due to higher microRNA levels in the modified mirVana method. Therefore, it was decided that both housekeeping genes would provide a more accurate normalization.

2. Validation study of tRF^{LysA} (1274A) and tRF^{LysB} (1274B) in Australian cohort

The levels of tRF^{LysA}, tRF^{LysB}, tRF^{LeuA} and tRF^{ThrA} were measured in 400 mesothelioma and control human serum samples. These tRFs had been identified in earlier studies to discriminate mesothelioma from normal and asbestos exposed individuals without disease. Comparison between mesothelioma and total controls and sarcoid mesothelioma compared with controls and lung cancer did not show any significant difference for any of the tRFs (tRF^{LysA} and tRF^{LysB} shown here). Further analysis is being performed to combine tRF data to identify any biomarker signature pattern.

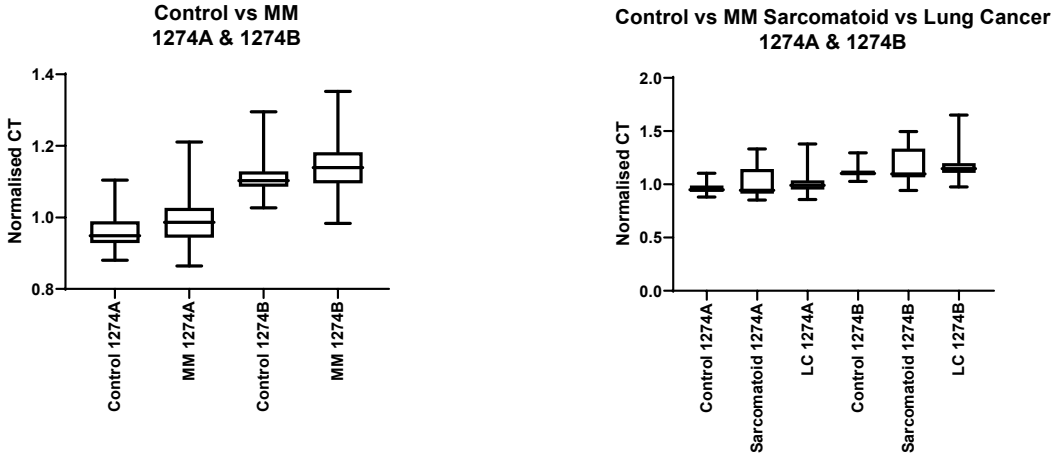

Figure 2. Validation study of tRF^{LysA} (1274A) and tRF^{LysB} (1274B) in Australian cohort does not support tRfs as biomarkers for mesothelioma

The levels of tRF^{LysA} (1274A) and tRF^{LysB} (1274B) were measured by real time PCR in 400 mesothelioma and control human serum samples and compared. Data comparing control vs mesothelioma and control vs sarcomatoid mesothelioma vs lung cancer did not show any significant difference.

3. The effect of time from collection on miRNA expression

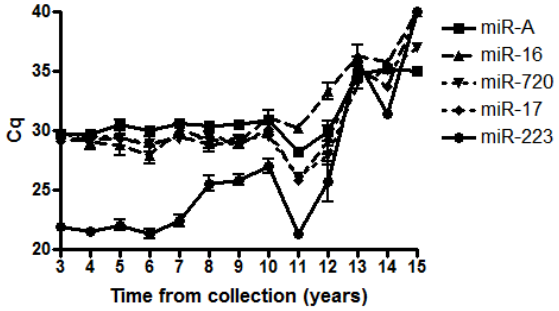

Figure 3. Real time PCR for miRNA (miR) in pooled serum samples from 3 patients with serum collected yearly for up to 15 years (run in triplicate). Data shows that there is a significant decrease in miRNA levels in 10 year old samples.

4. Expression of tRFs in human and mouse mesothelioma cell lines

The levels of tRF^{LysA}, tRF^{LysB}, tRF^{LeuA} and tRF^{ThrA} were measure in human and mouse cell lines using primers designed for human tRF sequences (n=4). Mouse tRF primers aren't available but given the high sequence homology between human and mouse tRFs, we initially tried the human primers. The levels of tRFs in human cell lines are higher than in mouse cell lines but this may be due to the primers we used. Primers have been designed based on mouse tRF sequences and will be assessed. Based on the results from this study we will use the human cell lines OLD1612 and STY51 for overexpression studies and VGE and JU77 cells for inhibition studies. Mouse cell lines for *in vivo* studies are yet to be confirmed.

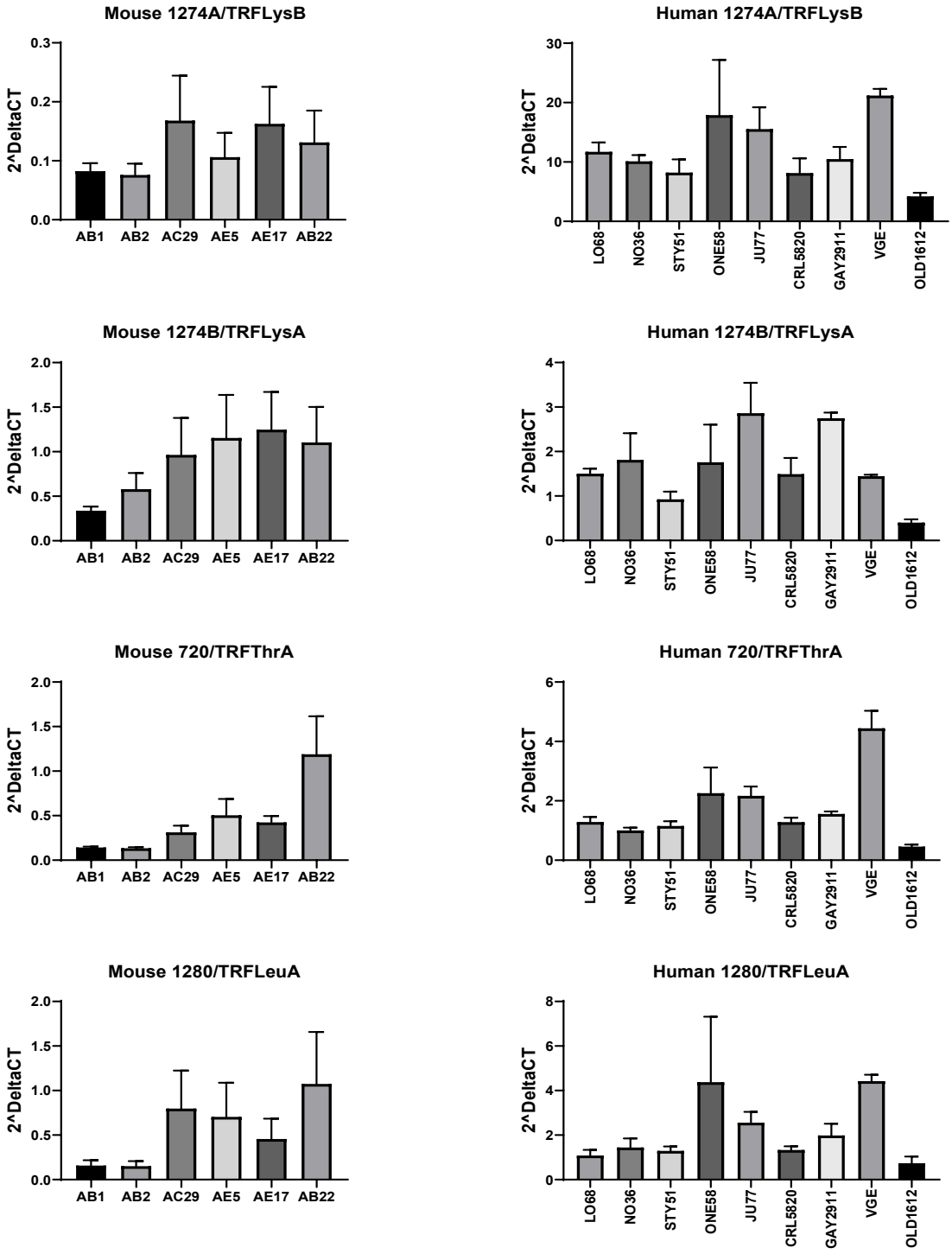

Figure 4. Expression of tRFs in human and mouse mesothelioma cell lines. The levels of tRF^{LysA}, tRF^{LysB}, tRF^{LeuA} and tRF^{ThrA} were measure in human and mouse cell lines. Expression levels for human cells were significantly higher than for mice.