



BRITISH LUNG FOUNDATION

Final Report

Please return to Ian.Jarrold@blf.org.uk

1. Grant-holder details

Grant Holder(s) and positions held	Professor Marija Krstic-Demonacos	Grant no	MESOUK16-4
Research worker(s)	Emyr Bakker, Parisa Meysami, Hasen Alhebshi		
Place at which research is carried out	University of Salford		

Grant details

Title of Research	Hypoxamirs as a New Target for Treatment of Malignant Pleural Mesothelioma (MPM)
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Registered charity in England and Wales (326730),

the Isle of Man (1177).

blf.org.uk

2. Report in lay language

3a. Lay title

Addressing Hypoxia as a New Target for Treatment of Asbestos-induced Cancer

3b. Lung diseases relevant to the research

3c. Summary - please write a 'lay abstract' of your work in language that a non-scientist can understand.

Focus on:

- Questions being addressed by the work and why the work was needed
- Describe how the work was carried out
- Describe the results found
- Describe how the results might help people with lung disease in the short/longer term

This section may be used as the basis of communications to the general public and MUST be understandable to a non-scientist. Please minimise the use of technical terms and explain those used. 500 words maximum

The role of hypoxia (low oxygen) in cancer has been extensively studied and this has disclosed intriguing insights into how it favours cancer development and progression. Malignant pleural mesothelioma (MPM) is a cancer related to asbestos exposure, which is known as a hypoxic tumour that is not effectively treated by chemotherapy. Hypoxia can induce changes in gene expression, and hypoxia causes MPM tissues to express high levels of "hypoxamirs". Hypoxamirs are a type of microRNAs (also called miRNAs or miRs), which are short RNA molecules that can alter the cellular levels and hence functions of factors important for the progression of the disease. These changes imposed by miRs can be assessed through measuring gene and protein levels of their targets. Exposure to asbestos affects levels of miRs in MPM cells, suggesting a role for miRs also in the development of MPM and further highlighting their importance. miRs are also currently being investigated as therapeutic targets in clinical settings for patients with MPM. Our preliminary results on cell lines and tissues from patients show that hypoxamir expression is related to worse prognosis. It has also been shown that altering levels of downregulated miRs affected growth of MPM cells.

The aim of our project is to comprehensively analyse the hypoxamirs function and explore their potential as therapeutic targets in MPM. MPM cell lines exposed to hypoxia and tissues obtained from MPM patients were used to monitor the levels of expression of miRs associated with hypoxic conditions as well as gene expression levels across the genome. Based on results identifying the specific subset of miRs and genes expressed under conditions mimicking those in patients, clinical trials targeting miRs and/or their targets can be planned with the ultimate aim of improving therapy of MPM.

DETAILED LAY REPORT

3d. Background / reasons for the research

Malignant Mesothelioma is a rare and aggressive disease that develops from the mesothelial cells of the serous membranes, with more than 80% of cases affected by malignant pleural mesothelioma (MPM). Mesothelioma is mostly caused by asbestos exposure with other factors including exposure to a simian virus 40 (SV40), radiation and erionite (a naturally occurring mineral similar to asbestos). There are 1000-3000 new cases of malignant mesothelioma per year depending on the country in question, with the peak number of cases in Europe estimated around 2020. MPM has a very poor prognosis and is a difficult cancer to treat, thus representing an unmet clinical need. Research studies conducted so far generally did not successfully translate to improved therapeutic outcomes. Therefore, innovative approaches, to identify novel, potentially successful drugs, are required.

Hypoxia is one of the main characteristics of this tumour contributing to bad prognosis and to resistance to chemotherapy. MPM cells express high levels of the hypoxia biomarkers that correlate with worse prognosis in MPM. HypoxamiRs are a group of microRNAs (miRs) controlled by hypoxia, that play a role in Epithelial Mesenchymal Transition (EMT, a process important for metastasis and cancer spread), chemo-resistance and are unfavourable prognostic factors. Preliminary results showed that in 7 MPM cell lines hypoxia affected the expression of miR-210 and that the potential hypoxamirs Let-7c-5p + miR-15a-5p are negative prognostic factors.

In this project we aimed to analyse miRNA expression in MPM cells in hypoxic conditions, in conjunction with whole genome expression data, to identify and validate their targets in MPM tissues, and to evaluate their value as therapeutic targets.

3e. What problem/need/question does this research address?

Hypoxia is a common characteristic of MPM, but its use in therapy has not been fully explored. Investigation of the role of hypoxia-modulated microRNAs (hypoxamiRs) in MPM will provide a deeper understanding of the hypoxic pathways in MPM. Characterising hypoxamiRs, their targets and related pathways can potentially lead to identification of novel therapeutic approaches, by identifying hypoxamiRs as relevant drug targets or through modulation of pathways controlled by hypoxamiRs.

3f. Can you summarise the need for your research in one sentence?

This research could lead to improved treatment of mesothelioma aimed to target hypoxamiRs and their targets in cancers where hypoxia is an important factor.

3g. How was the research carried out?

Multiple human MPM cell lines (PPM-Mill, REN, Phi, Rob) were used in this research. Cells were exposed to hypoxia to mimic the tumour environment and determine the global changes in gene (transcriptome) and miRNA expression (miRome). Targets of modulated miRNAs were identified through bioinformatics analysis and validated using quantitative Real Time- Polymerase Chain Reaction (qRT-PCR) and western blot analysis, molecular biology and biochemistry techniques which allowed for assessing individual gene and protein expression levels. Effects of relevant pathways on mesothelioma cells and hypoxia response were studied using assays to determine the effects of miRNAs overexpression (transfection of miR-210-mimic) or inhibition of its potential targets on cell survival

(Sulphorhodamine -SRB- assay) of mesothelioma cells. In addition, the biomarker potential and the clinical relevance of the identified pathways were investigated by immunohistochemistry via analysis of patient tissues.

3h. Please summarise the findings of your research project

In this project we identified changes in transcriptome and microRNA-ome caused by exposure of mesothelioma cells to hypoxia. Bioinformatics analysis was used to cross-reference transcriptome and microRNA-ome analysis and identify the subset of miRNAs and their targets selectively regulated by hypoxia. Several pathways potentially involved in hypoxic response in mesothelioma were identified and in particular the PGAM5 protein (PGAM family member 5, mitochondrial serine/threonine protein phosphatase) that is potentially being involved in the regulation of mitochondrial dynamics and acting as a central mediator for programmed necrosis. This protein is a target of miR210 that is increased in several mesothelioma cell lines exposed to hypoxia. miRNA210 target sequence is identified in PGAM5 and overexpression of a miR210 mimic resulted in the downregulation of PGAM5 mRNA and protein levels in mesothelioma cells. Luciferase constructs carrying PGAM5 target sequence also respond to miR210 mimic. These results confirm that PGAM5 is a mediator of hypoxia response and it is targeted by miR210 in mesothelioma cells. Immunohistochemistry based staining of clinical samples is under way to estimate the clinical relevance of these results.

3i. How do your research results contribute to or change the diagnosis, prevention, treatment and/or outcome of lung disease? Are there any impacts on quality of life? If there is no immediate impact for patients, do they have the potential for change in the future?

This research project is addressing a crucial factor that leads to resistance to therapy which is hypoxia and hypoxia related miRs and their targets. During this study novel microRNAs and their targets potentially involved in regulatory mechanisms leading to the hypoxic characteristics of MPM have been identified and have the potential to be prognostic markers. They can serve as starting points towards designing novel early phase clinical trials to test the clinical outcome of modulation of these pathways. Specific targeting of the hypoxic components of a particular tumour could lead to the development of personalised treatment of MPM patients. Given that the first line chemotherapy for MPM is not effective and that a second line treatment does not exist, the results of this project offer potential identification of novel therapeutic approaches and would be a step forward for the treatment of MPM patients.

3j. Has your work increased the understanding of lung disease? If so, how?

This research has increased understanding of mesothelioma disease. Project results include identification of novel hypoxia modulated miRs, associated pathways and their targets. These targets were characterised and are likely to provide further insight into the role hypoxia plays in disease development and progression as well as response to current therapy. Furthermore, these novel pathways can be studied further to determine their diagnostic and prognostic value as well as identify their potential as novel drug targets.

3k. Can you summarise the outcomes of your work in 3 bullet point 'take home' messages for our supporters? This should focus on why your work has been beneficial to people with lung disease.

Hypoxia is one of the main features of mesothelioma and one of the critical factors contributing to resistance to therapy. Increasing knowledge about this pathway will contribute to better understanding and therapy of this disease. The focus of this project was:

- Identification of transcriptome and microRNA-ome alterations in mesothelioma cells exposed to hypoxia.
- Determination of pathways relevant to hypoxic response in mesothelioma.
- Characterisation of newly identified pathways (PGAM5 as a target of miR210 and hypoxia)

3l. Developments/ new directions for research - what do you want to do next? Has this research resulted in further research funding?

The plan for further research includes completing publication process through involvement of PhD students that are currently in the laboratory and will help with revisions potentially needed in the publication process. Collaboration with Blackpool hospital has resulted in the award of the grant titled: "Identification of prognostic biomarkers and potential therapeutic targets in non-small-cell lung cancer and pleural mesothelioma" from the Rosemere Cancer Foundation. This will allow us to have access to patients' samples to study the potential value of the newly identified pathways as biomarkers.

Further grant applications are planned upon publication of the manuscript to validate our findings in 3D cellular models and translate findings into clinical settings.

3m. Glossary of terms used

Malignant Pleural Mesothelioma (MPM)

Hypoxia-related micro RNAs (hypoxamirs)

Transcriptome refers to global changes in gene expression

miRNome refers to global changes in micro RNA expression

3n. How has this research grant helped you? (e.g. influence on career progression, providing data to help you access further funding etc.) (in lay language)

This research grant has helped further career development of two young bright researchers who have, during research duration, secured prestigious positions (Emyr Bakker is now a Lecturer in UCLan and Parisa Meysami is a postdoc in the University of Bristol). Furthermore, a third researcher Hasen Alhebshi who was completing the immunohistochemistry part of the project is currently applying for biotechnology and NHS based positions. Several PhD students have been trained to use new techniques and have learned about the importance of mesothelioma. In parallel to this grant, further funding was obtained to analyse potential biomarkers in mesothelioma and enabled our group's access to patient samples and clinical data.

Are you happy for portions of this lay content to be used publically the BLF immediately?

If not, please provide a date when we can do so

NO -we are preparing this work for publication, and publishing it immediately would impair this process.

DATE: upon publication of the manuscript

4. Scientific outcomes of your research Please summarise the research findings from your project on not more than one side of A4 paper (single spaced, two sides if double spaced) (font size no less than 10).

To ensure the integrity of MPM cell lines and that they are responsive to hypoxia, we performed mycoplasma testing and analysed their miR-210 levels following exposure to hypoxia, using methodology that is employed by researcher groups in the hypoxia field. Numerous mesothelioma cell lines showed altered miR-210 levels in hypoxia with best response observed in REN cells that were chosen for further testing. REN cells were exposed to normoxia and hypoxia for 6 and 24 hrs, RNA was extracted and sent to the Molecular Biology Core Facility at the Cancer Research UK Manchester Institute. Data of miRNAs and mRNAs were analysed according to either < -1.5 or > 1.5 Log₂ fold change to select significantly altered miRNAs and mRNAs. Up-regulated miRNAs in hypoxia included hsa-miR-210, hsa-miR-200, hsa-miR-132-5p*, hsa-miR-132-3p and hsa-miR-135b; down-regulated hsa-miRNAs in hypoxia included hsa-miR-320a and hsa-miR-320b. Then, miRNA based quantitative RT-PCR (RT-qPCR) was performed to validate the miRNAs profiles in human mesothelioma cell lines. Multiple mesothelioma cell lines (REN, Phi, PPMill, Rob) were exposed to normoxia and hypoxia conditions for 6 and 24 hours, and total RNA extracted and subjected to miRNA quantification.

No significant changes in hypoxia treated cells were observed for the miR-135-5p whereas the miR-132-5p* was significantly upregulated by hypoxia in PPM-Mill cell line but in REN, Rob and Phi cell lines this upregulation was not statistically significant. miR-200 showed complex pattern of regulation by hypoxia across MPM cell lines, with most significant effect observed in PPM-Mill cell lines after 6 hrs of hypoxia. miR-320a validation showed downregulation by hypoxia in PPM-Mill cell line. Validation of hypoxamirs sequencing confirmed that only mir210 is robustly upregulated in all tested MPM cell lines and was therefore chosen for further analysis.

Potential miRNA target identification was carried out using publicly available software: TargetSCAN, DIANA and PicTar. mRNA levels determination revealed that ESCO2, CENPEN, XRCC5, BARD1, PGAM-5 were significantly downregulated in REN cell line after 24 hours in hypoxia. Further testing and validation that included downregulation of PGAM5 protein and mRNA levels in cells expressing the mir210 mimic as well as response of luciferase construct confirmed that PGAM5 is a target of mir210. Current analysis in the laboratory includes determination of cell death pathways and mitochondrial biogenesis in cells in which PGAM5 gene expression has been silenced using siRNA/mir210 mimic in the presence and absence of hypoxia. Finally, the potential clinical value of PGAM5 is being evaluated using immunohistochemistry to screen 100 slides from mesothelioma patients.

5. Problems encountered during the research. If the start date of your work was delayed, please clarify reasons.

During the course of the grant leading investigator Dr Luciano Mutti has left University and Professor Marija Krstic-Demonacos and Dr Gianpiero Di Leva have led grant to successful completion.

In addition, we had to employ 3 research technicians during duration of the grant as they have found other positions relevant for their career progression, therefore delaying executions of the grant aims.

6. Publications arising or planned from this grant (please enclose a copy of each) - please acknowledge the BLF in all publications that arise from our funding and forward copies of future publications

Original papers and publications

Manuscript is in preparation.

Abstracts presented at meetings

None.

I certify that to the best of my knowledge all the information given in this report is correct and I will continue to acknowledge the British Lung Foundation and keep them informed about future publications and developments arising from this work. Electronic signatures are acceptable.

Signed

Dr Diana Kuo-Dennis