THE UNIVERSITY OF NEWCASTLE

Investigating the Genetics of the Development of Lung Cancer

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A thesis submitted in fulfilment of the requirements

for the degree of Doctor of Philosophy in

Immunology and Microbiology

December 2020

STATEMENT OF ORIGINALITY

I hereby certify that the work embodied in the thesis is my own work, conducted under normal supervision. The thesis contains no material which has been accepted, or is being examined, for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made. I give consent to the final version of my thesis being made available worldwide when deposited in the University's Digital Repository, subject to the provisions of the Copyright Act 1968 and any approved embargo.

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Statement of Collaboration

I hereby certify that the work embodied in this thesis has been done in collaboration with other researchers, or carried out in other institutions. I have included as part of the thesis a statement clearly outlining the extent of collaboration, with whom and under what auspices.

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December 2020

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A. Acknowledgements

I owe my deepest gratitude to my primary supervisor Professor Philip Hansbro for providing me with the opportunity to undertake this PhD project under his guidance. I would also like to sincerely thank him and my co supervisor Dr Chantal Donovan as without their constant support, guidance and mentorship this PhD thesis would not have been possible. I would specially like to offer my sincere thanks and gratitude to Dr Chantal Donovan for genuinely caring for my research as well as for my mental well-being. I am truly grateful to Dr Nicole Hansbro for always being there for me, you have always been my point of contact when I was in trouble professionally and personally. Your constant support and help have really made this journey easier.

I would like to thank the core members of the Lung cancer team Priyanka Sahu and Sophie Pickles for their constant help, support and advice. You both have really been a strong pillar for me throughout my PhD. I would specially like to thank my dear friend Sophie Pickle, you have been my colleague, housemate and my constant support throughout my PhD. The number of times you have stepped up to ease my burden is countless and I am really grateful for it.

I would like to extend my heartfelt thanks to all the past and present members of Hansbro and Horvat group for accepting me as a part of this big amazing group. I wish to thank Dr Henry Gomez and Dr Kurtis Budden for unofficially mentoring me and teaching me all the skills I needed to complete my thesis. Your constant banter was a real stressbuster and made this journey so much fun. I would like to make special mention of Associate Professor Jay Horvat for his pep talks that helped me in stressful situations. I would specially like to thank Dr Bernadette Jones, Tegan Hunter, Dr Alexandra Brown, Dr Prema Mono Nair, Dr Tatt Jhong Haw, Lohis Balachandran and Dr. Richard for their help in endpoints and Nathalie Kiaos, Emma Bee, Bree Anderson, Bradley Mitchell, Carol Devine, Simon Gao for helping me with smoking protocol and animal monitoring. I would also like to thank all members of the Centenary UTS Centre for inflammation for their help and support during the last one year of my PhD.

I would sincerely like to thank all our collaborators, Dr Helle Bielefeldt- Ohmann for performing all the histology analysis, Dr Neil Watkins, Dr Parwinder Kaur, Dr Bhavna Hurgobin and Dr Alen Faiz for helping me with the whole genome sequencing analysis.

The acknowledgement wouldn't be complete without thanking the University of Newcastle for providing me with the opportunity to work in one of the best research institutes, Hunter Medical Research Institute. I would also like to thank the university for providing me with the scholarships and student funds that helped me survive this PhD. I would like to thank Maitland Cancer Appeal for providing additional funding that helped me to conduct my research. I would like to extend my sincere gratitude to Jennie Thomas for providing me with the prestigious Emlyn and Jennie Thomas Postgraduate Medical Research Scholarship which not only helped me to survive my PhD but also boost my morale.

Finally, I would like to thank the people who mean the most to me in my life. I wouldn't have been doing what I love if it was not for my parents and my sister, Karuna. Thank you all for always believing in me and encouraging me to follow my dreams even if that meant being thousands of miles away from you all. Your constant encouragement, understanding and pride has always lifted me in my lowest time. And a special thanks to my partner, Pritesh for being my solid rock specially during the last couple of weeks. Thanks for pushing me to do better and always having my back.

This year has been particularly stressful and difficult and I would like to once again thank each and every one mentioned above as this thesis wouldn't have been possible without your support.

B. Abstract

Background: Lung Cancer (LC) is one of the most commonly diagnosed cancers and is a leading cause of cancer related death worldwide. Cigarette smoking is the major risk factor responsible for development of LC. Despite the advances in cancer therapeutics, LC has a poor survival rate of ~15% over five years. The current image based diagnostic techniques detect LC when the tumour is already at an advanced stage or metastasised. Since we do not have the data on genetic alterations that takes place early in the development of LC (preneoplastic lesions), the currently available biomarker based diagnostic techniques also fail in early diagnosis. The main problem with obtaining data on genetic alteration for preneoplastic lesions is the difficulty in tissue collection from humans when the tumour is at early stages. However, since mouse models can be manipulated to develop different stages of LC, the tumour tissue can be collected at different stages and analysed to identify genetic alterations responsible for preneoplastic lesions.

Hypothesis and Aims: Our laboratory has previously developed a mouse models that develops bronchioalveolar adenoma (BAA) (early stage of LC) in response to cigarette smoke and tobacco carcinogen, 4-methylnitrosamino-3-pyridyl-1-butanone (NNK). We hypothesise that this mouse model could be used as reference to establish a clinically relevant mouse models that develop both BAA and bronchioalveolar carcinoma (BAC) (late stage of LC). Performing whole genome sequencing on tumours isolated from mouse model that develop BAC will enable identification of genetics alteration responsible for BAC. The validation of these genetic alteration in mouse models that develop BAA will further enable identification. of genetic alteration that occur early in development of LC.

Methods: The female A/J mice were treated with 2 carcinogens, cigarette smoke (CS) and NNK. The order of cigarette smoke exposure and NNK administration varied based on the mouse models. The carcinogen treatment was followed by an air recovery period. Histological analysis of the lung was assessed by staining lung sections with haematoxylin and eosin to determine the tumour type, tumour incidence and multiplicity. The airway inflammation was assessed by enumerating the inflammatory cells present in the bronchoalveolar lavage fluid that was collected and processed during the endpoint. Lung function was also analysed using the forced oscillation technique to determine the functional changes in the lung in response to CS exposure and NNK administration.

For genome analysis, whole genome sequencing was performed on DNA extracted from mouse model where NNK treated mice were exposed to CS for 36 weeks followed by an air recovery period of 27 weeks (3xNNK+36wk CS+27wk air reocery period) using Illumina NovaSeq 6000 platform. The resultant WGS data was analyed using bioinformatics tools.

Results: Mouse models where the female A/J mice were treated with 3 doses of NNK followed by 12, 18, 24 and 36 weeks of CS followed by 12, 18, 24 and 27 weeks of air recovery period respectively developed BAA. The mouse model where NNK (3 doses) treated mice were exposed to 24 and 36 weeks of CS followed by 24 and 27 weeks of air recovery period respectively also developed BAC along with BAA. The mouse model where NNK (3 doses) treated mice were exposed to 12 and 36 weeks of CS followed by 12 and 27 weeks of air recovery period showed 100% tumour incidence in 2 experimental groups, one treated with only NNK (NNK/Air) and other treated with both NNK and CS (NNK/CS). The NNK/CS-exposed mice showed a trend of higher tumour multiplicity as compared to the NNK/Air-exposed mice in these mouse models.

The WGS analysis identified 38 somatic mutations in 36 different genes that were common in tumours isolated from NNK/CS and Sal/CS-exposed mice. The genes identified in this analysis were found to be mutated in clinical samples of patients with BAC as seen in COSMIC database. The analysis of WGS data also revealed the mutational processes associated with tumours induced in NNK/CS and Sal/CS-exposed mice by generating mutational signatures. The mutational signature revealed that NNK was the major contributor of carcinogenesis in NNK/CS-exposed mice

The mouse models where female A/J mice were first exposed to CS followed by NNK administration and air recovery period developed BAA. The mouse model where mice where exposed to 8 weeks of CS followed by 3 doses of NNK and 8 weeks of air recovery period showed 100% tumour incidence in 2 experimental groups, one treated with only NNK (Air/NNK) and other treated with both CS and NNK (CS/NNK). The tumour incidence was reduced to 25% and 75% in Air/NNK and CS/NNK-exposed mice respectively in mouse model where mice were exposed to 8 weeks of CS followed by 1 dose of NNK and 8 weeks of air recovery period (8wk CS + 1xNNK + 8wk air recovery period). This model showed a significantly higher tumour multiplicity in CS/NNK-exposed mice as compared to Air/NNK-exposed mice. With increase in CS exposure to 12 weeks followed by 1 dose of NNK and 12 weeks of air recovery period model, the tumour incidence was increased to 87.5% in Air/NNK and CS/NNK-exposed mice. This model showed a trend of higher tumour multiplicity in Air/NNK as compared to CS/NNK-exposed mice.

Conclusion: When the A/J mice were first treated with NNK followed by CS exposure and air recovery period, the mice develop BAA which further progress to BAC with increase in CS exposure beyond 24 weeks in NNK/CS-exposed mice. However, the WGS analysis of tumours from 3xNNK+36wk CS+27wk air recovery period model revealed NNK as the major contributor of carcinogenesis. By exposing the mice first to CS followed by administration of reduced dose of NNK in 8wk CS + 1xNNK + 8wk air recovery period model the tumour multiplicity was increased in CS/NNK-exposed mice as compared to Air/NNK-exposed mice. This suggest that CS might be the major contributor of carcinogenesis in this model, however further analysis is required to confirm this.

C. Publications and funding related to this thesis

Publications:

1. Vamshikrishna Malyla, Keshav Raj Paudel, Shakti D Shukla, Chantal Donovan, Ridhima Wadhwa, Sophie Pickles, **Vrushali Chimankar**, Priyanka Sahu, Helle Bielefeldt-Ohmann, Mary Bebawy, Philip M Hansbro, and Kamal Dua. Recent advances in experimental animal models of lung cancer. Future Medicinal Chemistry 2020; 12: 567-570.

2. Gaetano Caramori, Paolo Ruggeri , Sharon Mumby , Antonio Ieni, Federica Lo Bello, **Vrushali Chimankar**, Chantal Donovan, Filippo Ando, Francesco Nucera, Irene Coppolino, Giovanni Tuccari, Philip Hansbro and Ian M. Adcock. Molecular links between COPD and lung cancer:new targets for drug discovery? Expert opinion on therapeutic targets 2019; 23:539-553

3. Bernadette Jones, Chantal Donovan, Gang Liu, Henry M. Gomez, **Vrushali Chimankar**, Celeste L. Harrison, Cornelis H. Wiegman, Ian M. Adcock, Darryl A. Knight, Jeremy A. Hirota and Philip M. Hansbro. Animal models of COPD: What do they tell us? Respirology.2016 Advance online publication. doi: 10.1111/resp.12908.

Poster presentations:

1. Vrushali Chimankar, Chantal Donovan, Sophie Pickles Priyanka Sahu, Parwinder Kaur, Bhavna Hurgobin, Alen Faiz, Helle Bielefeldt-Ohmann, Henry Gomez, Vamshikrishna Malyla, Peter Wark, Neil Watkins, Philip M. Hansbro. Can genomic

sequencing on the mouse model of lung cancer help us to understand the genetics of human lung cancer at: The Australian Lung Cancer Conference, 2020 19-21 February, Melbourne, Australia.

2. Vrushali Chimankar, Sophie Pickles, Chantal Donovan, Priyanka Sahu, Atiqur Rahman, Henry Gomez, Vamshikrishna Malyla, Helle Bielefeldt-Ohmann, Peter Wark, Neil Watkins, Philip M. Hansbro. Using experimental mouse models to understand the pathogenesis of lung adenocarcinoma at: Hunter Cancer Research Symposium, 2019 November 8; Newcastle, Australia.

3. Vrushali Chimankar, Sophie Pickles, Chantal Donovan, Priyanka Sahu, Atiqur Rahman, Henry Gomez, Vamshikrishna Malyla, Helle Bielefeldt-Ohmann, Peter Wark, Neil Watkins, Philip M. Hansbro. Can experimental mouse models help us to understand lung cancer pathogenesis? at: 6th Annual ASMR Hunter Region Satellite Scientific Meeting, the 3rd of June, 2019, Newcastle, Australia

4. Vrushali K Chimankar, Celeste L Harrison, Atiqur Rahman, Sophie Pickles, Priyanka Sahu, Helle Bielefeldt-Ohmann, Peter Wark, Neil Watkins, Philip Hansbro.Mouse models to investigate the genetic mechanism underlying the development of Non-small cell lung cancer at: Hunter Cancer Research Symposium, 2018 November 1; Newcastle, Australia.

5. Vrushali K Chimankar, Celeste L Harrison, Atiqur Rahman, Sophie Pickles, Priyanka Sahu, Helle Bielefeldt-Ohmann , Peter Wark, Neil Watkins , Philip Hansbro.Mouse models to investigate the genetic mechanism underlying the

development of Non-small cell lung cancer at: Sydney Cancer Conference 2018 October 11-12; Newcastle, Australia.

6. Vrushali K Chimankar, Celeste L Harrison, Atiqur Rahman, Sophie Pickles, Priyanka Sahu, Helle Bielefeldt-Ohmann , Peter Wark, Neil Watkins , Philip Hansbro. Investigating the Genetics of the Development of Lung Cancer. Poster session presented at: Hunter Cancer Research Symposium, 2017 November 24; Newcastle, Australia.

Funding Related to this thesis

Project title: Identification of genomic mutations associated with the development and progression of lung cancer for use in early diagnosis

Sponsor/scheme: Cancer Council NSW/Research Grant

Funding period: 2016-2020

Total funds awarded: $360,000

D. Abbreviations

ADC	Adenocarcinoma
ALK	Anaplastic lymphoma kinase
AMPK	Amp activating protein kinase
BAA	Bronchioalveolar adenoma
BAC	Bronchioalveolar carcinoma
BALF	Bronchoalveolar lavage fluid
BAP	Benzo(a) pyrene
BRAF	V-raf murine sarcoma viral oncogene homolog b
CDKN2A	Cyclin-dependent kinase inhibitor 2a
CS	Cigarette smoke
CT	Computerised tomography
DSS	Disease specific survival
EGFR	Epidermal growth factor receptor
FEV	Forced expiration volume
GDP	Guanosine diphosphate
GWAS	Genome-wide association study
HER2	Human epidermal growth factor receptor 2
IVC	Individually ventilated cages
KRAS	V-kiras2 kirsten rat sarcoma viral oncogene homolog
LC	Lung cancer
LKB1	Liver kinase b1
LOH	Loss of heterozygosity
MAP2K1	Mitogen-activated protein kinase kinase 1
MAPK	Mitogen-activated protein kinase
MS	Mainstream smoke
MDSC	Myeloid-derived suppressor cells
NGS	Next generation sequencing
NHMRC	National health and medical research council
NK	Natural killer
NNK	4-methylnitrosamino-3-pyridyl-1-butanone
NNN	N'- nitrosonornicotine

OCT	Optimal cutting temperature
OS	Overall survival
PAH	Polycyclic aromatic hydrocarbons
PBS	Phosphate-buffered saline
PFS	Progression free survival
PJS	Peutz-jeghers syndrome
RB	Retinoblastoma
RET	Rearranged during transfection
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SNP EFFECT	Snpeff
TAMs	Tumour-associated macrophages
TH	Helper t
TILS	Tumor infiltrating lymphocytes
TM	$Cd45ro^{+}$ memory cells
TN	Tumour nest
TP53	Tumour protein 53
TREGS	Regulatory t cells
TS	Tumour stroma
WGS	Whole genome sequencing

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