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FINAL REPORT

Project / Program Title		Platelet functional defects caused by mutations of transcription factor GFI1 B: a new mechanism of human disease
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Award Received		2015 RACP Fellows Research Entry Scholarship
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Administering institution		Kolling institute of Medical Research
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PROJECT SUMMARY

Platelets mediate clot formation after tissue trauma. inherited platelet disorders (IPDs) comprise a heterogeneous group of disorders characterized by platelet functional defects and/or low platelet counts (inherited thrombocytopenia (IT)). individuals with these disorders may experience bleeding symptoms which vary in severity, moreover, it is becoming increasingly recognised that in some cases, these disorders predispose individuals to cancers (haematological as well as solid organ malignancies).

Platelets are made in the bone marrow, production of these cells are controlled by a tightly regulated group of genes and proteins, called transcription factors. in 2013 our group at the Northern Blood Research centre, Kolling institute of Medical Research, University of Sydney was the first to describe an IT caused by mutation in a transcription factor called GFI18. individuals in this large multi-generational family experienced severe (but variable) bleeding and had characteristic features (large platelets with a heterogeneous reduction in important platelet granules) on the blood film that distinguished this disorder form other platelet conditions.

Our project aimed to ascertain the frequency of GFI1 B related thrombocytopenia (GFI18-RT) in our community and explore the mechanisms underpinning GFI1 B-RT. A better understanding of the molecular pathogenesis of these disorders enables one to design better methods of detection, treatment and ultimately work towards disease correction through targeted gene modification strategies using platforms such as CRISPR/Cas9 (which we are exploring in our laboratory).

We performed genetic analysis using a platform called, next generation sequencing, for individuals referred to our laboratory with uncharacterised thrombocytopenia. This platform was extremely successful in providing numerous families with a diagnosis of their platelet conditions to a molecular level. We also detected additional mutations causing GFI18-RT which we further explored in the laboratory.

In the laboratory, we used a revolutionary process whereby skin cells obtained from patients with GFl18-RT were reprogrammed into cells with stem cell like properties, called induced pluripotent stem cells (iPS cells). These cells are then changed (differentiated) into functional human platelets. Through this process we were able to explore the mechanisms underpinning diseases from the earliest precursor cell, the stem cell, into the final functional cell that is in the bone marrow and blood. This technique, coupled with other laboratory assays allowed us to characterise two additional variants of GFl1 B affecting different functional regions of the gene that resulted in different clinical bleeding patterns. We described the likely mechanism (alteration of downstream gene promoters). Furthermore, we identified a common feature of increased CD34 expression on platelets of all affected individuals with GFl18-RT to date, and we proposed this be used as a simple laboratory assay that could be employed to screen for GFl18-RT.

PROJECT AIMS / OBJECTIVES

l) Ascertain the prevalence of GFl1B-AT in the Australasian community

- We employed next generation sequencing (NGS) platform using a candidate gene panel.

- This Identified additional families with GFI 1 B-RT secondary to variants affecting different functional domains of the transcription factor.

2) Assess the efficacy of NGS lo diagnose an inherited platelet disorders that remained uncharacterised despite traditional phenotypic testing.

- We performed genetic analysis on -120 individuals from 13 centres ln the Australasian region. We Identified the causative gene mutation in a significant portion of these individuals including variants in MYH9 which Is associated with nephritis, deafness and cataracts and three families with RUNX1 mutations. These individuals have predisposition to acute myeloid leukaemia and therefore an ability to detect these variants in of obvious Importance.

3) Explore the mechanism underpinning thrombocytopenia and altered granule production in individuals with GFl1 B-RT

-We established an IPS cell platform and were able to recapitulate aspects of the clinical phenotype in these cells. This was extremely valuable in demonstrating

Increased CD34 by megakaryocytes and platelets in individuals with these variants. We propose that increased C034 detected by low cytometry can be employed as

a screening tool for the presence of GFl18 mutations.

- We used luciferase assays 10 demonstrate the action of GFl18 variants on GFl1 B promoter targets, and confirmed that C034 and TU881 were targets for GFl18.

·We used western blot and electrophoretic mobility shift assays to demonstrate altered DNA binding capacity of various GFl1B variants. This ls of significance when

considering the mechanism of action and interaction between transcription factors that are Important in megakaryopolesis.

4) Gene editing using CRISPR/Cas9

·We have begun to use this technology to "knock in" and ·correct" mutations in vitro.

SIGNIFICANCE AND OUTCOMES

1) Next generation sequencing

We have demonstrated that this platform Is efficacious for the diagnosis of inherited thrombocytopenia. This is of extreme value as many of these disorders are frequently misdiagnosed as immune thrombocytopenia with resultant inappropriate treatment.

Our success has promoted the expansion of our project around Australasia to include additional centres. We have also established a platelet co-operative group in

Sydney that incorporates this method. albeit. on a research platform/basis.

2) Our work has expanded knowledge regarding GFl1B-AT:

·We have demonstrated that the phenotype Is dependent on the functional domain perturbed by the mutation. This has Importance when diagnosing individuals with these mutations as knowledge of genotype-phenotype relationships enables patient and family education. prognostication and provides guidance for peri-operative treatments.

·We have shown that increased platelet sur1ace expression of CD34 detected by now cytometry can be employed as a screening lest indicating mutation in GFl18.

Flow cytometry Is performed during the investigation of inherited platelet disorders and Is a test employed by our co-operative initiative. It is therefore extremely valuable lo be able add this test to the panel of lest that hopefully translate into Improved diagnosis of these disorders.

3) Methods developed during our research:

- IPS cell platform development and utilisation - our expertise in this area Is can be applied 10 other projects and provides us the opportunity to share our experience and knowledge with other investigators in Australia. I have also had opportunity to learn further techniques from our collaborators in Cambr1dge (Cambridge University) and Japan (Kyoto). These protocols have potential for disease modelling purposes as well as wider application in blood transfusion services.

·We explored the use of mean platelet diameter measurements for the classification and diagnosis of inherited thrombocytopenia. Our findings were consistent with an Italian co-operative that has utilised this methodology. This can be used as an additional screening tool for inherited thrombocytopenia.

·We introduced and optimised immunofluorescence staining for the diagnosis of MYH9 related disorders into our laboratory. This has widely been accepted as a "gold standard" diagnostic test for MYH9 related disorders. As a consequence of the various MYH9 mutations detected by our NGS sequencing we sought to employ immunofluorescence staining and now offer this as an additional research tool to our referring haematologists.

Future work:

GFl18-An understanding of transcription factors that regulate haematopoiesis has broader application in the development of treatment strategies for these disorders

and therefore future work will focus on GFl18 interactions with other transcription factors that are important in haematopoiesis, and will we will explore epigenetic regulators and their interactions with GFJ1 B ·

IPS models -We plan to continue developing IPS cell platforms for disease modelling.

NGS- Further work and refinement of candidate gene panels may enable its utility in routine clinical laboratories. We have established collaborations with large International groups (Cambridge University and Birmingham University) with interests this field and will aim to expand and Improve our service in this area.

PUBLICATIONS / PRESENTATIONS

Publications:

1) 2017 Rabbolini DJ, Morn~Kopp, Chon a. et al., Thrombocytopenia end CD34 expression is decoupled from a-granule deficiency with mutation of the first GF11B zinc finger.

- Under revision Journal of Thrombosis and Haemostasis.

2) 2017 Rabbolini DJ, Mu11doll 8J, Gabriel S, et. Al. Receptor homodimerisation plays a critlcal role in a novel dominant negative P2RY12 variant identified in a family with severe bleeding

• Receptor homodimerisation critical role in a novel dominant negative P2RY12 variant identified in a family with severe bleeding.

In Review. Journal of Thrombosis Haemostasis

3) 2017 Ro. Rabbolini DJ, Chun Y, Latimer M. et al.

Diagnosis and treatment of MYH9-RD In an Australasian cohort with thrombocytopoenia

- In Press: Platelets Journal

4) 2017 Rabbolini DJ. E.E. Gardnier, Morel~Kopp MC et al

Anti-Glycoprotein VI mediated ITP: An under recognised significant entity

- In Press: Research and Practice in thrombosis and haemostasis (RPTH)