UNIVERSITI PUTRA MALAYSIA

DETECTION OF CHROMOSOMAL TRANSLOCATION [T(12;21)] AND REGULATORY T CELLS IN ACUTE LEUKAEMIAS USING FLOW CYTOMETRY

SITI ZULEHA BINTI IDRIS

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By

SITI ZULEHA BINTI IDRIS

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Master of Science

December 2013

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master in Science

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December 2013

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Leukaemia is a haematological malignancy detected in blood and bone marrow. Children achieve a 10-year survival rate of more than 85% but the prognosis for adult leukemia remains poor. Due to the heterogeneity of this disease a combination of laboratory techniques is needed for the classification and diagnosis of leukemias including cell morphology, cytochemistry, immunophenotype and cytogenetics. Cytogenetics provide information on treatment outcome. It is also a specific and personalized method for monitoring of minimal residual disease. The first chromosomal abnormality recognized was t(9;22) or BCR/ABL in chronic myeloid leukaemia (CML) by Nowell and Hungerford in 1959. Today more than 50 translocations have been reported and are essential in diagnosing and monitoring treatment outcome in leukaemia. Current methods used to detect and identify chromosomal translocations have some disadvantages. Conventional karyotyping method is a laborious procedure, insensitive (inadequate metaphase spreads, poor chromosome morphology) and need skilled technical staff whereas fluorescent in situ hybridization (FISH) and array comparative genomic hybridization (aCGH) are expensive tests. Polymerase chain reaction (PCR)-based kits are available however do not provide information on individuals cells of a mixed population, typical of clinical samples. Flow cytometry is a powerful equipment for rapid and multiparametric analysis of single cells providing both quantitative and qualitative information on size, internal complexity of cells and expression of markers by fluorescence labeled specific antibodies. Currently its usage is limited to determining lineage specificity of leukaemias (i.e. antigen expressions) while treatment monitoring (minimal residual disease) involves a panel of markers which overlap

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with normal blasts. The principle purpose of minimal residual disease is the detection of a rare population of leukemia blast whether immediately after treatment (for possible drug resistance) or after remission (for impending relapse). This will provide guidance on treatment. In-situ reverse transcriptase-polymerase chain reaction (RT-PCR) is a technique where cDNA is amplified within the cell. By combining these two methods and use of fluorescent primers, it is possible to detect chromosomal translocations within single cells using flow cytometry. The multiparametric potential of flow cytometry also allows the detection of rare cell populations with complex phenotypes such as regulatory T cells (Tregs). Tregs function by downregulating immune activation. However, this may also suppress the cancer immunosurveillance processes. Significantly increased numbers of Tregs has been reported in various cancers and has been suggested as a possible aetiology of acute leukaemias. Objectives of this study were to establish a method for in situ RT-PCR to detect chromosomal translocations in leukaemia and determine Tregs percentages using flow cytometry. Eighty acute leukaemia samples (44 acute myeloid leukaemia, 34 acute lymphocytic leukaemia, two mixed acute leukaemia) were screened for t(12;21) and t(8;21) using reverse transcriptase polymerase chain reaction (RT-PCR) method. Fifteen or 18% acute leukaemia samples were positive for t(12:21) and eight were positive in ALL cases. The t(8;21) translocation was detected in only two (2.5%) AML samples but in none of the ALL or mixed leukaemias. Twelve (80%) of the positive t(12;21) samples were analyzed using in situ RT-PCR and showed 80-97% positive cells with fluorescence phosphorammidite (FAM) labeled primers and detected by flow cytometry. Three colour staining was then performed by adding leukaemia markers (CD45PerCP and CD19PE or CD13PE) after in situ RT-PCR method which identified 50-90% positive cells. Tregs identified by the CD3+CD4+CD25+CD127- phenotype was found to be significantly higher in ALL samples (17 ALL samples) when compared to normal (30 normal healthy samples). In situ RT-PCR method may help in the investigation, diagnosis and monitoring of leukaemia by detecting chromosomal translocations specific to the patient. Multiparametric staining with markers of interest will further identify specific leukaemia populations and provide additional information that may help in disease management. In situ RT-PCR flow cytometry techniques have potential to be further developed and implemented in pathology services. Tregs may be important in immunosurvellance and cancer escape mechanism (pathogenesis of acute leukaemias) and could be used as targets for alternative therapy.

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Abstrak thesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk Ijazah Sarjana Sains

PENGESANAN TRANSLOKASI KROMOSOM [T(12;21)] DAN SEL PENGAWALATUR T PADA LEUKAEMIA AKUT MENGGUNAKAN SITOMETRI ALIRAN

Oleh

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Leukaemia adalah kanser darah yang dikesan di dalam darah atau sum-sum tulang. Kanak-kanak mempunyai 85% peluang untuk mencapai jangka hayat kehidupan selama sepuluh tahun atau lebih, tetapi prognosis bagi orang dewasa adalah kurang memuaskan. Gabungan kaedah diagnosis adalah perlu untuk mendiagnosa penyakit ini melalui kaedah morfologi sel, pewarnaan sitokimia, immunofenotip dan sitogenetik. Kaedah sitogenetik memberikan maklumat tentang keputusan rawatan. Ia juga spesifik dan akhirnya menjurus kepada diagnosis secara peribadi untuk memantau penyakit minimal residual. Ketidaknormalan kromosom yang pertama iaitu t(9;22) atau BCR/ABL pada pesakit leukaemia kronik myeloid telah dilaporkan oleh Nowell dan Hungerford pada tahun 1959. Sehingga kini lebih daripada 50 kromosom translokasi yang penting dalam diagnosis dan pemantauan yang telah dilaporkan. Kaedah sekarang untuk mengesahkan diagnosis dan pemantauan rawatan mempunyai beberapa kelemahan. Menkariotip secara konvensional adalah teknik yang sukar, tidak sensitif (metafasa yang tidak cukup, mutu morfologi kromosom yang tidak baik) dan memerlukan kakitangan makmal yang terlatih dimana bagi ujian *fluorescent in situ hybridization* (FISH) dan *array comparative genomic hybridization* (aCGH) adalah ujian yang mahal. Kaedah kit reaksi rantai polymerase (PCR) ada dikomersilkan, namun maklumat tentang individu sel dalam yang mana adalah tipikal untuk pada sampel klinikal, tidak dapat diperolehi. Sitometri aliran dalah peralatan yang berkeupayaan tinggi untuk mengesan dengan cepat dan melakukan analisis pelbagai parameter pada setiap sel dan boleh memberikan maklumat secara kuantitatif dan kualitatif untuk saiz sel, dalaman sel yang kompleks

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serta secara ekspresi penanda pendaflour yang dilabel spesifik dengan monoklonal antibodi. Pada masa sekarang, penggunaannya terhad kepada mengesan *lineage* spesifik pada leukaemia dan bagi memantau rawatan leukaemia (minimal residual disease) yang bagaimanapun melibatkan penanda panel yang bertindih dengan sel darah normal yang tidak matang. Prinsip penyakit residual minimal adalah mengesan populasi yang jarang pada sel darah yang tidak matang leukaemia sama ada selepas rawatan (kemungkinan kerintangan pada dadah atau ubat) atau selepas peredaan (untuk mengesan kambuhan penyakit). Ini akan membantu dalam pengurusan rawatan. In situ RT-PCR pula adalah kaedah di mana cDNA diamplifikasi di dalam sel. Dengan menggabungkan dua kaedah ini dan menggunakan primer pendaflour maka pengesanan ketidaknormalan kromosom boleh dilakukan di dalam sel. Potensi sitometri aliran dalam mengesan banyak parameter membolehkan ia mengesan populasi sel yang jarang dengan fenotip yang kompleks seperti sel pengawalatur T (Tregs). Tregs berfungsi dengan mengurangkan keaktifan sistem imun. Walau bagaimanapun ia turut menyekat imuno-pantauan kanser. Secara signifikan peningkatan Tregs dilaporkan dalam pelbagai kanser dan ia telah dikenal pasti sebagai salah satu penyebab kepada leukaemia akut. Objektif kajian ini adalah untuk memperkenalkan kaedah RT-PCR in situ untuk mengesan ketidaknormalan kromosom dalam leukaemia dan mengesan peratusan Tregs menggunakan sitometri aliran. Lapan puluh sampel leukaemia akut (44 leukaemia akut myeloid, 34 leukaemia akut lymphoid, dua leukaemia akut campuran) diperiksa untuk t(12;21) menggunakan kaedah reaksi rantai polymerase transcriptase membalik (RT-PCR). Lima belas atau 18% sampel leukaemia akut positif untuk t(12;21) dimana lapan adalah kes ALL. Bagi t(8;21) dikesan dalam dua sampel AML (2.5%) dan tiada kes yang positif bagi ALL atau leukaemia campuran. Dua belas sampel (80%) kes positif t(12;21) diuji menggunakan kaedah in situ RT-PCR dan menunjukkan 80-97% sel positif t(12;21) dengan menggunakan primer pendaflour *phosphorammidite* (FAM) yang dikesan oleh sitometri aliran . Kaedah pewarnaan tiga warna dilakukan dengan menambah penanda leukaemia (CD45PerCP dan CD19PE atau CD13PE) selepas kaedah in situ RT-PCR dan menunjukkan 50-90% sel positif. Tregs dikenalpasti dengan fenotip CD3+CD4+CD25+CD127- dan didapati lebih tinggi secara signifikan dalam ALL (17 sampel ALL) berbanding sampel normal (30 sampel individu normal). Kesimpulannya, RT-PCR in situ boleh membantu dalam penyiasatan, diagnosis dan pemantauan rawatan leukaemia dengan mengesan translokasi kromosom spesifik pada pesakit. Analisis pelbagai parameter dengan penanda yang tertentu akan dapat mengenalpasti populasi spesifik leukaemia. Ini akan memberikan maklumat tambahan yang dapat membantu dalam rawatan pesakit. Teknik RT-PCR in situ sitometri aliran mempunyai potensi untuk dibangunkan dan digunakan dalam perkhidmatan patologi. Tregs penting dalam imuno-pantauan, patologi leukaemia akut untuk membolehkan mekanisme kanser dikecualikan daripada imuno-pantauan dan mungkin boleh digunakan sebagai sasaran dalam terapi alternatif.

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I certified that a Thesis Examination Committee has set on 6th December 2013 to conduct the final examination of Siti Zuleha binti Idris on her thesis entitled "Detection of Chromosomal Translocations [T(12;21)] and Regulatory T Cells in Acute Leukaemias Using Flow Cytometry" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U. (A) 106] 15 March 1998. The Committee recommends that the student be awarded the Master of Science degree.

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LIST OF ABBREVIATIONS

ABL	Abelson murine leukemia viral oncogene homolog
aCGH	array comparative genomic hybridization
ALL	Acute lymphoblastic leukaemia
AML	Acute myeloid leukaemia
AML1	Acute myelogenous leukaemia 1
APC	Antigen presenting cells
ATCC	American Type Culture Collection
BBMC	Bone marrow mononuclear cells
BLAST	Basic local alignment search tool
BMA	Bone marrow aspirate
BCR	Breakpoint cluster region
bp	Base pair
CD	Cluster of differentiation
cDNA	Complementary DNA
CLL	Chronic lymphoblastic leukaemia
CLL	Chronic myeloid leukaemia
CSF	Cerebrospinal fluid
DEPC	Diethylpyrocarbonate
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EtBr	Ethidium bromide
FAB	French-American-British
FAM	fluorescent phosphorammidite
FISH	Fluorescence in-situ hybridization
HIV	Human immunodeficiency virus
HPV	Human pappiloma virus
JOE	6-carboxy-4', 5'-dichloro-2', 7'-dimethoxyfluorescein
LUX	Light upon extension
MDS	Myelodysplastic syndromes
$MgCl_2$	Magnesium Cloride
MGG	May-Grünwald/Giemsa
MMLV	Moloney murine leukaemia virus
MOH	Ministry of Health
MRD	Minimal residual disease
mRNA	Mesengger RNA
NCBI	National Center for Biotechnology Information
PAS	Periodic acid-Schiff
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
qPCR	Real time PCR
RNA	Ribonucleic acid

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RPMI	Roswell Park Memorial Institute
RT	Reverse transcription
RT-PCR	Reverse transcriptase PCR
TEL	Translocation-Ets-leukamia
Tregs	Regulatory T cells
WBC	White blood cells
WHO	World Health Organization

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CHAPTER 1

INTRODUCTION

Leukaemia is a haematological malignancy which originates from the bone marrow. It is caused by neoplastic proliferation of haematopoietic cells. It is divided into acute and chronic leukaemia of myeloid and lymphoid lineages. Acute leukaemia is characterized by a rapid increased in the numbers of immature blood cells and immediate treatment is needed to control the diseases as without treatment it will lead to death (Chandrasoma and Taylor, 1998; Bain, 2010).

The common clinical manifestations of acute leukaemia are anemia, thrombocytopenia and recurrent infections. Patients with anemia present with pallor and lethargy. Thrombocytopenia may lead to clinical features like spontaneous bruises, purpura, bleeding gums and menorrhagia (Hoffbrand and Moss, 2011).

Survival rate of acute leukaemia is dependent on age of the patients. Young and adolescent ALL show resistant to standard intensive chemotherapy. But as reported by Pui, 2009 steady progress was observed for both children and adolescents ALL of 5years free survival rates of about 79-86%. While, in AML, survival rates of young adults with complete induction therapy may reach 65-85% compared to older adults (Sekeres, 2008). For peadiatric AML, with lack of targeted therapy, 60% of survival rates were seen in clinical trials in developed countries.

Since the 1960s, many studies were done to understand the developments and etiologies of leukaemia which may be caused by ionizing radiation, chemicals, virus as well as chromosome abnormality (Shafer, 1965). Deoxyribonucleic acid (DNA) is replicated during cell division and provides the template for the process of translation to amino acid. If the process is faulty and result in genetic aberration, it may lead to formation of cancer including leukaemia. In 1959, the first chromosomal abnormality was reported by Nowell and Hungerford which was translocations t(9:22) or BCR/ABL in chronic myeloid leukaemia (CML). Following that, many researchers placed their interest in this matter and found many more translocations. Today, more than 50 translocations have been reported and are essential in diagnosing and monitoring of leukaemia. Furthermore, in the new classification of leukaemia by WHO (2008), genetic abnormality is one of the criteria needed for the diagnosis of leukaemia. The seven most common translocations in leukaemia are t(9:22), t(4:11), t(1:19), t(15:17), t(8:21), t(12:21) and inv16 which are also used in prognostication. Prognostication is crucial in the management of patients with leukaemia as it is used as an indicator of patient respond to therapy and clinical outcome as well as risk of relapse (Lo-Coco et al., 2003).

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Current methods available in the clinical laboratory to detect chromosomal abnormalities are conventional karyotyping, fluorescent in situ hybridization (FISH), reverse transcriptase polymerase chain reaction (RT-PCR) and array comparative genomic hybridization (aCGH). Conventional karyotyping is the gold standard in most genetic units in analyzing chromosomal abnormality. However this method is laborious, insensitive because of inadequate metaphase spreads, provide poor chromosome morphology, time consuming as several days is needed for cells to be incubated and need technically skilled staff. FISH and aCGH are expensive tests requiring new expertise and additional equipments. Polymerase chain reaction (PCR)-based kits are available however do not provide information on individuals cells of a mixed population, typical of clinical samples. New diagnostic tools and methods which utilize existing equipments, identify specific markers and can provide additional information will be beneficial in improving prognostication and disease monitoring.

Since the invention of the PCR technique in 1980's, this technique has become one of the best tools to detect chromosomal abnormalities as it not only detects specific gene but is also a sensitive method. In many laboratories, standard reverse transcriptase-polymerase chain reaction (RT-PCR), multiplex PCR, and real time PCR have been used widely in diagnosing leukaemia. The PCR method however, does not allow detection of chromosomal translocation within a single cell. Flow cytometry is now one of the most powerful equipment that is available. Most of the research or diagnostic laboratories have this equipment. Applications of flow cytometry are broad and its unique character is that it analyzes single cells, with rapid detection and multiparametric analysis. With flow cytometry, both qualitative and quantitative results are available. Currently its usage is limited to determining lineage specificity of leukaemia while treatment monitoring (minimal residual disease) involves a panel of markers which overlap with normal blasts. In-situ PCR amplifies cDNA within the cells. The combination of flow cytometry with RT-PCR potentially allows in-situ RT-PCR amplified of chromosomal translocation to be rapidly detected within cells with multiparametric possibilities.

In situ RT-PCR flow cytometry techniques have been reported in various studies for example in detection of viral DNA sequences, endogenous DNA sequences and as well as chromosomal translocations (Komminoth and Long, 1993). With the combination of these two methods, chromosomal translocations detected in acute leukaemia samples may be used in investigating, diagnosis, monitoring and minimal residual disease detection in leukaemias. This in situ RT-PCR flow cytometry may help in management and treatment of patients by providing effective and cheaper cost and also could give results with good turnaround time. In this study, t(12;21) and t(8;21) were chosen because these are common translocations found in acute leukaemia patients and associated with a favourable prognosis.

Elucidation of mechanisms and pathways in the investigation of pathogenesis and drug resistance in cancers are involving more complex and rare cell populations.

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Regulatory T cells or Tregs were discovered in the early 80's to 90's and have the function to down-regulate and control immune response (Sakaguchi et al., 2007). Tregs make up 5-10% of positive CD4 mature T cells. This population of cell is identified by the combination of monoclonal antibodies CD4 and CD25 and more recently with negative expression of CD127. The requirement of multiparametric detection and being a rare population make flow cytometry the ideal method for its identification. Wu et al. (2012) by using flow cytometry method reported increased Tregs in acute lymphoblastic leukaemia (ALL) and may play a significant role in tumor cells escaping immunity, leading to the immune tolerance of tumors. This study has not been substantiated by others.

Objectives of this study are:

1. To screen for chromosomal translocations t(8;21) and t(12;21) in acute leukaemia samples using standard reverse transcriptase polymerase chain reaction (RT-PCR).
2. To establish a multiparametric *in-situ* RT-PCR method for detection of chromosomal translocation t(12;21) and surface markers in a leukaemia cell line using flow cytometry.
3. To detect chromosomal translocation, t(12;21) in acute leukaemia samples using *in-situ* RT PCR-flow cytometry.
4. To determine percentage of Tregs in blood samples of acute leukaemia patient and apparently healthy individuals by flow cytometry.

Hypotesis of this study are:

1. In-situ RT-PCR method on flow cytometry allows multi-parametric detection of surface markers and chromosomal translocation in leukemia samples.
2. Tregs population is increased in acute lymphoblastic leukemia samples compared to healthy controls.

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