UNIVERSITI PUTRA MALAYSIA

EXPRESSION OF POTENTIAL STEM CELL-RELATED MARKERS IN ACUTE MYELOID LEUKAEMIA CELL LINES AND GENE SILENCING EFFECT OF HOXA3 IN THP-1 CELLS

HINDHUJA PANNEERSELVAN

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By

HINDHUJA PANNEERSELVAN

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EXPRESSION OF POTENTIAL STEM CELL-RELATED MARKERS IN ACUTE MYELOID LEUKAEMIA CELL LINES AND GENE SILENCING EFFECT OF HOXA3 IN THP-1 CELLS

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Leukamia is a type of malignancy which originates in the bone marrow where haematopoiesis takes place. Acute myeloid leukaemia (AML) is one subtype which is more common among adults. AML is a heterogeneous disease marked by poor prognosis, high disease-related mortality and frequent relapse. Leukemic stem cells (LSCs) may play a role in early relapse of the disease. However, the LSC phenotype and regulatory mechanism that confer a higher resistance of LSCs to treatment is still unclear. Characterization of LSC associated biomarkers in available AML cell lines and related control mechanisms will provide an opportunity to use these as disease models to understand the biological characteristics of LSC and its relation with treatment outcome. The aim of this study was to characterize the LSC by phenotype and function. RT-qPCR was used to analyze the gene expression levels of a limited number of published LSC biomarkers (*ALDH*, *IL3RA*, *CLEC12A*), 86 new identified genes and 37 unidentified genes (chromosome contig) from an earlier study as well as standard markers associated with early haematopoiesis (*CD34* and *CD38*) and myelopoiesis (*ANPEP*/CD13, *Siglec-3*/CD33 and *CD14*) on myeloid leukaemia cell lines: K562, THP-1 and HL-60 and induced pluripotent cells from THP-1 (iPSC-THP-1). Due to similarity in phenotype to human embryonic stem cells as reported, iPSC-THP-1 cells were used as reference to identify the patterns of stem cell related markers. Flow cytometry method was used to confirm the phenotype of a selected number of these markers including ALDH, *IL3RA*/ CD123, *CLEC12A*/CD371, HOXA3, ENPP4, *ANPEP*/CD13, CD34, CD38 on the cell lines in addition to another myeloid leukaemia cell line, KG-1a and cord blood cell samples. *HOXA3* from the poor prognosis library was selected for siRNA gene silencing study in THP-1. Lipofectamine 2000 reagent was used to transfect siRNAs into THP-1 and cells were allowed to incubate for 72 hours. Subsequently, intracellular and surface marker staining were performed using flow cytometry to determine the silencing effect of *HOXA3* on THP-1 cell. All samples examined were positive for the combined CD13/CD14/CD33 markers confirming myeloid origin. The cell lines were double

positive for $CD34^{+}CD38^{+}$except iPSC-THP-1 which were mainly $CD34^{+}CD38^{-}$ and cord blood which were $CD34^{+}CD38^{low}$. Stem cell feature of iPSC-THP-1 was confirmed with positivity for ALDH, which was also expressed on HL-60, the most mature cell lines. *IL3RA*/CD123 was lower in iPSC-THP-1 and cord blood and higher in other cell lines but was absent in HL-60. Expression of *CLEC12A*/CD371 was also absent only in very early stem cells (iPSC-THP-1) and mature cells (HL-60) but expressed earlier in cord blood cells. ALDH+ expression appeared inverse to *IL3RA*/CD123 and *CLEC12A*/CD371. Based on these, predicted cells with decreasing stem cell characteristics were iPSC-THP-1, cord blood cells, other myeloid leukaemia cell lines (THP-1, K562, KG-1a) and HL-60. HOXA3 appeared to increase with cell immaturity with highest expression in iPSC-THP-1 (RT-qPCR result) followed by cord blood cells and THP-1 while ENPP4 was reversed and highly expressed on HL-60. Both were absent in K562. These two genes were selected from an earlier study from this group. Genes identified from RT-qPCR with potential for gene silencing study were *HOXA3, SF3B1, NBPF15* and *MTND5*. THP-1 was selected as potential leukaemia stem cell line for further gene silencing with *HOXA3*. Gene silencing resulted in reduction in expression of *HOXA3* as well as other markers (CD14, CD34, CD38, CD123 and CD371) post transfection. The $CD34^{+}CD38^{-}$ phenotype was expressed only on very early stem cells (iPSC THP-1). The published stem cell biomarkers, ALDH was positive on iPSC-THP-1 but not CD123 and CD371 which appeared only in later progenitor cells. The combined expression of $HOXA3^{+}ENPP4^{+/-}$ may be as useful as $CD34^{+}CD38^{-}$ to use as cut-off to differentiate LSCs from early progenitor cells. Knockdown experiment with HOXA3 suggested its importance in maintaining stem cell character. These results require validation in primary AML samples.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk Ijazah Master Sains

EKSPRESI SEL STEM YANG BERPOTENSI BERKAITAN PENANDA MYELOID LEUKEMIA CELL LINES DAN KESAN GENE SILENCING HOXA3 DI THP-1

Oleh

HINDHUJA A/P PANNEERSELVAN

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Leukamia adalah sejenis kanser darah yang berasal dari sum-sum tulang di mana haematopoiesis berlaku. Leukaemia Myeloid Akut (AML) adalah salah satu subjenis yang lebih biasa di kalangan orang dewasa. AML adalah penyakit *heterogeneous* yang ditandai dengan prognosis yang buruk, kadar kematian yang tinggi dan berulang. Sel stem leukaemia (LSCs) berperanan dalamrelaps awal penyakit. Walau bagaimanapun, fenotip LSC dan mekanisme pengawalseliaan LSC yang memberi rintagan LSC yang lebih tinggi kepada rawatan masih tidak jelas. Karakterisasi biomarker berkaitan LSC dalam *cell line* AML yang tersedia dan mekanisme kawalan yang berkaitan akan memberi peluang untuk menggunakannya sebagai model penyakit untuk memahami ciri-ciri biologi LSC dan kaitannya dengan hasil rawatan. Tujuan kajian ini adalah untuk mencirikan LSC dengan fenotip dan fungsi. RT-qPCR digunakan untuk menganalisis tahap ekspresi sejumlah kecil biomarker LSC yang diterbitkan (ALDH, CD123, CD371), 86 gen yang baru dikenal pasti dan 37 gen yang tidak dikenali (*chromosome contig*) dari kajian sebelumnya serta penanda standard yang berkaitan dengan haematopoiesis awal (CD34 dan CD38) dan myelopoiesis (*ANPEP*/CD13, *Siglec-3*/CD33 dan CD14) pada *cell line* leukaemia myeloid: K562, THP-1 dan HL-60 dan sel pluripoten yang disebabkan dari THP-1 (iPSC-THP-1). Oleh kerana persamaan dalam phenotype kepada sel stem embrio manusia seperti yang dilaporkan, *cell line* iPSC-THP-1 digunakan sebagai rujukan untuk mengenal pasti pola yang berkaitan dengan sel induk penanda. Kaedah *Flow cytometry* digunakan untuk mengesahkan fenotip bilangan penanda ini yang dipilih termasuk ALDH, *IL3RA*/ CD123, *CLEC12A*/CD371, HOXA3, ENPP4, *ANPEP*/CD13, CD34, CD38 pada *cell line* selain satu lagi *cell line* leukaemia myeloid, KG-1a dan *cord blood cell*. HOXA3 dari *poor prognosis* dipilih untuk kajian silencing gene siRNA dalam THP-1. Reagen Lipofectamine 2000 digunakan untuk memeindahkan siRNAs ke THP-1 dan sel-sel dibenarkan untuk inkues selama 72 jam. Selepas itu, pewarnaan penanda intraselular dan permukaan dilakukan menggunakan *flow cytometry* untuk menentukan *gene silencing* HOXA3 pada sel THP-1. Semua sampel yang diperiksa adalah positif untuk gabungan penanda CD13 / CD14 / CD33 yang mengesahkan asal myeloid. *Cell line* leukaemia myeloid yang dinilai positif dua kali ganda untuk $CD34^{+}$ $CD38^{+}$ kecuali

iPSC-THP-1 dengan majoriti sel $CD34^{+}CD38^{-}$ dan *cord blood cell* yang $CD34^{+}$ $CD38^{low}$. Ciri sel induk iPSC-THP-1 disahkan dengan positif untuk ALDH, yang juga dinyatakan pada HL-60, *cell line* yang paling matang. IL3RA/CD123 adalah lebih rendah dalam iPSC-THP-1 dan *cord blood cell* dan lebih tinggi dalam *cell line* lain tetapi tidak ditemui di HL-60. Ekspresi *CLEC12A*/CD371 serupa, tidak ada hanya pada sel stem awal iPSC-THP-1) dan sel matang (HL-60) tetapi dinyatakan sebelum dalam *cord blood cells*. $ALDH^{+}$ muncul terbalik kepada ekspresi *IL3RA*/CD123 dan *CLEC12A/*CD371. Berdasarkan ini, sel-sel yang diramalkan dengan ciri-ciri sel stem yang berkurangan adalah iPSC-THP-1, *cord blood cells*, cell line leukaemia myeloid lain (THP-1, K562, KG-1a) dan HL-60. HOXA3 nampaknya meningkat dengan ketidakmatangan sel dengan ekspresi tertinggi dalam iPSC-THP-1 (hasil RT-qPCR) diikuti oleh *cord blood cell* dan THP-1 sementara ENPP4 dibalikkan dan sangat dinyatakan pada HL-60. Kedua-duanya tidak hadir di K562. Gen yang dikenal pasti dari RT-qPCR yang berpotensi untuk kajian *gene silencing* adalah HOXA3, SF3B1, NBPF15 dan MTND5. THP-1 dipilih sebagai *stem cell line* leukaemia berpotensi untuk *gene silencing* selanjutnya dengan HOXA3. *Gene silencing* mengakibatkan pengurangan ekspresi HOXA3 serta penanda lain (CD14, CD34, CD38, CD123 dan CD371) selepas transfeksi. Fenotip $CD34^{+}CD38^{-}$ dinyatakan hanya pada sel stem yang sangat awal (iPSC-THP-1). Biomarker *stem cell* yang dicadangkan ini, ALDH positif pada iPSC-THP-1 tetapi bukan CD123 dan CD371 yang muncul hanya dalam sel-sel progenitor kemudian. Ekspresi gabungan HOXA3+ENPP4+/- mungkin berguna sebagai CD34+CD38- untuk digunakan sebagai cut-off untuk membezakan LSC dari sel-sel progenitor awal. Eksperimen Knockdown dengan HOXA3 mencadangkan kepentingannya dalam mengekalkan watak sel stem. Keputusan ini memerlukan pengesahan dalam sampel AML utama.

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This is to confirm that:

- the research conducted and the writing of this thesis was under our supervision;
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LIST OF ABBREVIATIONS

AGO2	Argonaute 2
ALL	Acute Lymphoblastic Leukeamia
ALDH	Aldehyde dehydrogenase
AML	Acute Myeloid Leukaemia
APC	Allophycocyanin
APL	Acute Promyelocytic Leukaemia
ASO	Antisense oligonucleotides
BAALC	Brain and Acute Leukaemia, Cytoplasmic
BLAST	Basic local alignment search tool
BM	Bone marrow
BMA	Bone marrow aspirate
BMT	Bone marrow transplantation
bp	Base pair
cDNA	Complementary DNA
CEBPA	CCAAT enhancer-binding protein
CLEC12A	C-type lectin domain family 12 member A
CLL	Chronic Lymphocytic Leukaemia
CML	Chronic Myeloid Leukaemia
CN-AML	Cytogenetically normal acute myeloid leukaemia
CR	Complete remission
CSC	Cancer Stem Cell
C_T	Threshold cycle
DEPC	Diethylpyrocarbonate
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DsRNA	Double-stranded RNA
EDTA	Ethylenediaminetetraacetic acid
EtBr	Ethidium bromide
FAB	French-American-British
FBS	Fetal Bovine Serum

FITC	Fluorescein Iso-thiocyanate
FLT3	Fms-like tyrosine kinase 3
GP	Good Prognosis
HCT	Hematopoietic Cell Transplantation
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HOXA3	Homeobox protein Hox-A3
HSCs	Hematopoietic Stem Cells
IFN	Interferon
IL3Rα	Interleukin 3 receptor, alpha
IMDM	Iscove's Modified Dulbecco's Medium
iPSC	Induced Pluripotent Stem Cell
LPC	Leukaemia Progenitor Cells
LSC	Leukaemia Stem Cells
MAbs	Monoclonal Antibodies
MDS	Myelodysplastic Syndromes
MFC	Multiparameter Flow Cytometry
miRNA	MicroRNA
MRD	Minimal Residual Disease
mRNA	messenger RNA
MTND5	Mitochondrially Encoded NADH Dehydrogenase 5
NBPF15	Neuroblastoma breakpoint family, member 15
NOD/SCID	Nonobese diabetic/severe combined immunodeficiency
NPM1	Nucleophosmin 1
PBS	Phosphate buffer saline
PCR	Polymerase Chain Reaction
PE	Phycoerythrin
Per CP	Peridine and Chlorophylprotein
PKR	Protein Kinase R
PP	Poor Prognosis
RISC	RNA-induced silencing complex
RNAi	RNA interference
RPMI	Roswell Park Memorial Institute
RT-PCR	Real Time PCR

RT-qPCR	Reverse-Transcription quantitative Polymerase Chain Reaction
SCID	Severe Combined Immunodeficiency
SD	Standard deviation
SEM	Standard error of mean
SF3B1	Splicing factor 3b subunit 1
siRNA	Small interfering RNA
TRM	Treatment Related Mortality
WBC	White blood cells
WHO	World Health Organization

CHAPTER 1

INTRODUCTION

1.1 Background of the study

Leukaemia is a hematological disease where patients present with an abnormal number of immature white blood cells in the bone marrow, where new blood cells are generated (Colmone *et al.*, 2008). The bone marrow typically consists of a small number of immature cells or sometimes called as blast cells under normal conditions. In leukaemia, these blast cells undergo a malignant change which then divide in an uncontrolled way and function abnormally. Leukaemia blast cells invade the blood rapidly then eventually spread to other parts of the body through blood circulation and the lymphatic system to other organs such as liver, spleen and lymph node (Hope *et al.*, 2004).

Leukaemia can be either myeloid or lymphocytic referring to the cell types in which the leukaemia was initiated. Myeloid leukaemias develop from early cells that may give rise to monocytes, eosinophils, basophils and neutrophils, and possibly red blood cells as well as platelets, while lymphocytic leukaemias develop from cells that give rise to either T-lymphocytes, B-lymphocytes or natural killer (NK) cells. Certain subtypes of leukaemia are more common in different age groups although both children and adults have possibility of developing either types of leukaemia (Jordan *et al*., 2002). Leukaemia can be further divide into two types which are acute and chronic. Each leukaemia type is termed depending on how fast the disease develops. In acute leukaemia type, the affected bone marrow generates excessive number of abnormal blast cells and these leukemic cells will then accumulate in bone marrow and restrict the normal blood cells production (Reya *et al.*, 2001). Acute leukaemia develops rapidly and hence needs to be treated immediately after diagnosis.

Acute myeloid leukaemia (AML) is a heterogeneous clonal disease reported by accumulation of undifferentiated myeloid blast cells. The disease course is marked by poor prognosis, high disease-related mortality and frequent relapse. Former clinical studies have focused on the identification of prognostic subgroups in adult AML with the aim of guiding patients into risk-adapted therapies. These studies confirmed that cytogenetic abnormalities are prognostic, some favorable and other unfavorable and these are an integrated part of international recommendation in classification and diagnosis of acute myeloid leukaemia by World Health Organisation (WHO) (Hope *et al*., 2004; Guan *et al*., 2000; Guzman *et al*., 2001).

Leukaemia stem cells (LSCs) are characterized by their ability for self-renewal and the capability to produce clonogenic leukemic progenitors, which eventually generate huge numbers of blast cells. These LSCs are enriched in a $CD34^{+}CD38^{-}$

subpopulation, and successively give rise to $CD34^{+}CD38^{+}$ leukaemia progenitor cells (LPC) that further differentiate into the $CD34^{-}$ leukemic blast population (Jordan, 2002). Current anti-proliferative chemotherapeutic drugs usually target the rapidly clonogenic progenitors to achieve disease remission (Dick, 2008). However, quiescent AML LSCs are not effectually eliminated by existing treatments which may be reason that lead to some patients relapse early while others survive longer. Therefore, it is important to develop novel therapeutic agents to eradicate AML LSCs by aiming the self-renewal pathways of LSCs without affecting normal hematopoietic stem cells (HSCs) (Jin *et al.*, 2006).

A previous study was carried out to compare gene expression in patients of good (disease free survival >12 months) and poor prognosis (disease free survival < 12 months) and identified various potential leukaemia stem cell associated genes (Ngiow *et al.*, 2016). Further examination of these markers and the mechanisms involved in regulation of leukaemia stem cells are essential to identify candidate prognostic genes and its potential use in AML treatment since patient response to treatment is heterogeneous and the survival rate remains poor. Besides, in some patients no prognostic markers exist to predict outcome of the conventional therapy.

In this research project, myeloid leukaemia cell lines were characterized for expression of the genes and phenotyped for selected biomarkers. Gene silencing using siRNA inhibition experiments was conducted on a myeloid leukaemia cell line that expressed the selected gene to unravel its potential functions involved in LSCs. Leukaemia cell lines are considered as attractive models aimed to better characterize LSC biological properties because LSCs are relatively infrequent in patient's bone marrow, therefore they are difficult to be isolated and identified. Screening of prospective cancer related genes in cell lines may facilitate the identification of cancer targets which useful in revealing potential targets for cancer drugs and associated biomarkers. Elucidation of gene expression pattern could help to reveal the pathogenic mechanisms involve in AML, prognosis and risk categories (Obulkasim *et al.*, 2017). This research will characterize LSC associated biomarkers in available cell lines to recognize the biological characteristics of LSC that may be useful to guide treatment and predict outcome.

1.2 Problem Statement

Leukaemia stem cells (LSC) have been demonstrated to be associated with poor prognosis in AML. Nevertheless, LSC associated biomarkers and the LSC phenotype are still unconfirmed for diagnosis and clinical application. These are essential to predict and guide the outcome of treatment. Existing clinical classification uses markers from genetic abnormalities including chromosome translocations and mutations. However, these markers are appropriate only to 60% of acute leukaemia while the rest carry normal cytogenetics. Due to that, *in vitro* models essential for evaluation and mechanistic investigation of LSCs are still unavailable. Various potential LSC biomarkers in association with acute myeloid leukaemias (AML) have been published, however, have not been used to identify LSC cells in commercially

available myeloid leukaemia cell lines which are known to be of different stages of immaturity. The functional role of these biomarkers in the regulation of LSC remains elusive. Hence, expression of selected LSC associated genes/proteins in myeloid leukaemia cell lines and function of the biomarkers are worth investigating.

1.3 Objectives

The general objective of this research is to investigate the gene and protein expression of putative prognostic markers in AML cell lines and iPSC-THP-1 cells.
The specific objectives of this research study are:

1.) To evaluate the immunophenoype of AML cell lines and iPSC-THP-1 cells using flow cytometry.
2.) To investigate the effects of HOXA3 gene knock-down on the expression of LSC surface markers in THP-1 parental cells.

1.4 Hypothesis

We hypothesised that:

1.) Potential stem cell associated markers selected from earlier published studies will provide a relevant resource to identify a potential LSC in vitro model among AML cell lines.
2.) Gene silencing of a selected biomarker in AML cell line will reveal its potential function in leukaemia stem cell and as a target for alternative therapy.

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