Original article

Gene expression profiling in normal cytogenetic acute myeloid leukaemia of Malay patient

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Abstract

Background: Insight into recent molecular analyses of patients with acute myeloid leukaemia (AML) and a normal cytogenetic have revealed a striking heterogeneity with regard to the presence of acquired changes in gene expression. Nano String technology which is aimed to identify descriptive profiling of normal cytogenetic AML in differentially expressed genes involved in the different pathways in normal cytogenetic AML patients. ***Materials and method:*** Blood samples were obtained from eight at diagnosis patients with normal cytogenetics AML, two follow-up patients and two normal healthy controls prior to RNA extractions. RNA gene expression assay was performed using Nano Stringn Counter® Pan Cancer Pathway Panel (Nanostring Technologies, Seattle, Washington). Briefly, 100 ng of each total RNA sample was used as input into then Counter Pan Cancer Pathway Panel sample preparation. Data was extracted using then Counter RCC Collector and raw data output was imported into nSolver v2.6 analysis software for data analysis. ***Results:*** The age range was from 13-69 years, with a median age-range of 41 years. We found the most enriched up regulated genes in newly diagnosed normal cytogenetic AML enlisted MPO (7.25 log FC), FLT3 (5.02 log FC), MYCN (4.99 log FC), MYB (4.74 log FC), ITGA9 (4.48 log FC), KIT (4.41 log FC), MCM2 (3.47 log FC), RAD51 (3.40 log FC), CCNA2 (3.37 log FC) and PROM1 (3.24 log FC) which those commonly be found in AML cases. For highly expressed down regulated genes were GZMB (-5.80 log FC), IL8 (-5.79 log FC), TNFRSFSF10C (-5.03 log FC), LEF1 (-4.82 log FC), IL2RB (-4.70 log FC), IL7R (-4.44 log FC), BCL2A1 (-4.15 log FC), RASGRP1 (-4.13 log FC), IL1R2 (-4.00 log FC) and HSPA6 (-3.99 log FC).***Discussion and conclusion:*** Nano String required smaller amounts of starting material, and can perform mRNA expression profiling with digital precision, therefore the results do not require further validation by another method. MPO expression was significantly associated with disease-free survival (DFS). Previous report demonstrated that the groups by the MPO expression in the intermediate cytogenetic risk group showed a significant difference in DFS (p<0.001). A study by Valk et al., (2004) showed FLT3, a hematopoietic growth factor receptor, is the most common molecular abnormality in AML. The presence of such mutations in FLT3 and elevated expression of the transcription factor EVI1 confer a poor prognosis. N-MYC expression levels in AML samples from patients with favorable, intermediate, or unfavorable prognosis were compared with that in CD34+ cells from four healthy bone marrow donors. The most highly down regulated gene in newly diagnosed AML goes to Granzyme B (GZMB) which involved in cytolytic activity that showed high correlation with other transcripts expressed in activated cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells as well as lymphocyte activation-related gene validating it as a robust and specific metric of active cellular immunity. Increased expression of IL-8 and/or its receptors has been characterized in cancer cells, endothelial cells, infiltrating neutrophils, and tumor-associated macrophages, suggesting that IL-8 may function as a significant regulatory factor within the tumor microenvironment.

Keywords: N*ormal cytogenetic AML; gene expression*

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Introduction

Acute myeloid leukaemia (AML) is the diversified disorder which is caused by the accumulation of abnormal white blood cells in the bone marrow. AML also caused by the formation of myeloid cells when blood cells are not able to produce granulocytes or monocytes. These myeloid cells will form blast cells by differentiating into haematopoietic progenitor cells that clumps together. Several disease such as anaemia, blood clotting and severe infection developed from the accumulated blast cells in the bone marrow caused death from AML. In human T-cell, there are three types of oncogenes that are responsible for AML which is human T-cell lymphotropic viruses type I and type II (HTLV-1, HTLV-2) and simian T-cell. These oncogenes forms natural killer T cell complex (NKT) which kills the target cells major histocompatibility complex (MHC) class I and class II. Besides this, genes involved in inhibition of T-cell proliferation are also known to cause AML[1].

Although both lower and higher percentages have been reported, the proportion of adults with cytogenetically normal AML has varied from 40% to 49% in the cytogenetic studies[2]. The contradictory among studies could relate to some factors including the number of older patients studied, as the proportion of cytogenetically normal cases increases with age[3]. These cytogenetically normal patients have been classified in an intermediate-risk prognostic based on complete remission (CR) rate, relapse risk, and survival are poorer than those of sufficiently treated patients with such favourable aberrations as t(8;21)(q22;q22), inv(16)(p13q22)/t(16;16)(p13;q22), or t(15;17)(q22;q21) but better than those of patients with unfavourable cytogenetic outcomes such as inv(3)(q21q26)/t(3;3)(q21;q26), balanced translocations involving 11q23 other than t(9;11)(p22;q23), or a complex karyotype.

The microarray technology study on AML patients is beneficial in identification of genomic mutations. The identification of various up regulated and down regulated genes such as PML, TNFα, JUNB, FOS, TP63, VEGFA and ID2 can be determine by microarray gene expression profiling[1]. In Nano String, cytogenetic analysis of patients with AML led to identification of 133 genes. A new molecular profiling technology like NanoString can generate precise genomic information from small amounts of fixed patient tissues. This platform used digital, colour-coded barcodes or code sets attached to sequence-specific probes, permitting quantification of mRNA expression. Then Counter System have been used in recent studies for prognostic and predictive investigation in chemotherapy trials[4,5]and randomised placebo-controlled studies[6].

Insight into this study, Nano String technology have been used to identify descriptive profiling of normal cytogenetic AML in differentially expressed genes involved in different pathways.

Methods

Sample collection

Blood samples were obtained from eight at diagnosis patients with normal cytogenetics AML, two follow-up patients and two normal healthy controls. The diagnosis of normal cytogenetic AML was made based on morphology and immunophenotyping. This study was approved bythe Human Research Ethics Committee UniversitiSains Malaysia (USM/JEPeM/15090292).

Total RNA extraction and quality assessment

Prior to extraction, the work surface and pipettes were wiped with RNAse ZAP™ to promote an RNAse free environment. Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions including the on-column DNasetreatment. Total RNA concentration and RNA ratios (260/230 and 260/280) were determined by using NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Inc., Waltham, MA, USA). The RNA integrity was confirmed with the Agilent 2100 bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). The RNA integrity number (RIN) was calculated for every case and the RIN more than 7 was considered for downstream analysis. Samples that showed RNA degradation (RIN <7) were excluded from this study. After the quality of total RNA was assessed, samples were then stored at -80^{0}C until further analysis. Prior to analysis, samples were thawed on ice.

Nano Stringn Counter Pan Cancer Pathway Panel for gene expression profiling

RNA gene expression assay was performed using NanoStringnCounter®PanCancer Pathway Panel (Nanostring Technologies, Seattle, Washington). Briefly, for nCounterPanCancer Pathway Panel sample preparation, 100 ng of each total RNA sample

was used as input. Hybridisation of the samples were performed for 16 hours at 65°C. Then, the strip tubes were transferred tothe nCounter Prep Station for automated sample purification and subsequent reporter capture. Each sample was scanned for 280 FOV on the nCounter Digital Analyzer. Data was extracted using the nCounter RCC Collector and raw data output was imported into nSolver v2.6 analysis software.

Data analysis

Normalisation of each sample was done according to the geometric mean of the top 100 most highly expressed mRNAs. The mean plus twice the standard deviation of the eight negative controls for each sample was subtracted from each mRNA count in that sample. Further downstream analysis only included mRNAs with non-negative counts across all samples.

The mean of the negative controls was calculated by adding 2SD to the mean of the negative controls (threshold = mean + 2SD) as it functioned as the medium stringency threshold for mRNA detection and high stringency (NanoString Technologies nCounter Expression Data Analysis Guide).

Ethical clearance:

This study protocol has been granted approval for implementation by the Jawatankuasa Etika Penyelidikan Manusia Universiti Sains Malaysia (JEPeM-USM) and has been assigned study protocol code USM/JEPeM/15090292

Results

Basic characteristics of the newly diagnosed normal cytogenetic AML patients

Blood samples from 8 normal cytogenetic AML patients at diagnosis contributed 3 females and 5 males and 2 normal cytogenetic AML post treatment patients 1 male and 1 female and normal subjects; 1 male and 1 female1 (Table 1). The age range was from 13-69 years, with a median age-range of 41 years.

Table-1 Patient demographic and clinical characteristics.

Characteristics	AML patients, n= 8
Age, years	
Median	41
Range	13-69
Sex – no. (%)	
Female	3 (37)
Male	5 (63)
Haemaglobin (g/dL)	
Median	8.35
Range	6.6-12.9
Platelet count (10^9/µl)	
Median	45.88
Range	17-93
White cell count (10^9/µl)	
Median	30.22
Range	4-109.33

Differentially expressed genes in newly diagnosed AML with normal cytogenetics using NanoStringnCounterPanCancer Pathway assays

A total of 770 of gene profiles were detected using the NanoStringnCounter assay above background across all samples and 40 housekeeping genes. The dataset was retained and experienced $\log_2$ transformation prior to statistical analysis.

For raw data normalisation, internal positive spike controls was used to account for variability in the hybridization process. The data was further normalized to the average counts of all endogenous mRNA genes in each lane to account for any variability in the sample input. The gene profiles detection was determined using a metric that yields at a confidence level of 95% ($p<0.05$). This finding measured all genes in which the count of the mRNA was two standard deviations above the average of negative spike probes.

Table-2 The differentially expressed genes in newly diagnosed normal cytogenetic AML patient

Up regulated Genes	Log Fold Change	Down regulated Genes	Log Fold Change
MPO	7.25	GZMB	-5.80
FLT3	5.02	IL8	-5.79
MYCN	4.99	TNFRSFSF10C	-5.03
MYB	4.74	LEF1	-4.82
ITGA9	4.48	IL2RB	-4.70
KIT	4.41	IL7R	-4.44
MCM2	3.47	BCL2A1	-4.15
RAD51	3.40	RASGRP1	-4.13
CCNA2	3.37	IL1R2	-4.00
PROM1	3.24	HSPA6	-3.99

Heatmap for the differentially expressed genes

A heat map of the data is provided in Figure 1. Genes and samples were structured by hierarchical clustering, and coloured bars show each the value of each sample for each covariate. Each row is a single gene, and each column is a single sample. Sample names will be illegible in large datasets, in which casenSolver's interactive heat map functionality to provide a more view.

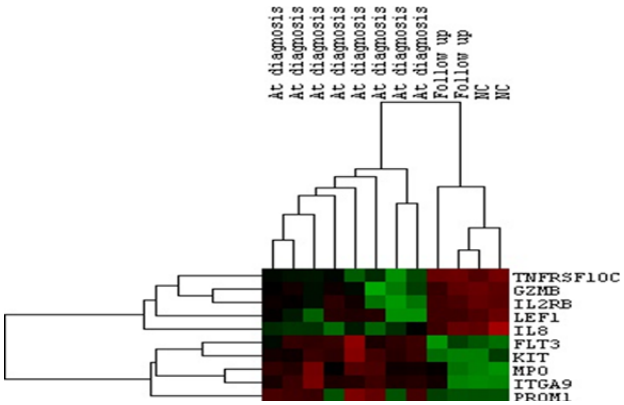

Fig. 1. Heatmap of each column represents one GC sample with red (up regulated), green (down regulated) and black (no change in expression).

In microarray analysis, the hierachical clustering based heatmap is a convenient method[7]. The heat map was designed for differentially expressed genes based on their statistical values. The heat map shows probe intensities for each gene probe id indicating the up regulated (red) and down regulated (green) genes respectively. These expressions level are measured based on logFC value and P-value.

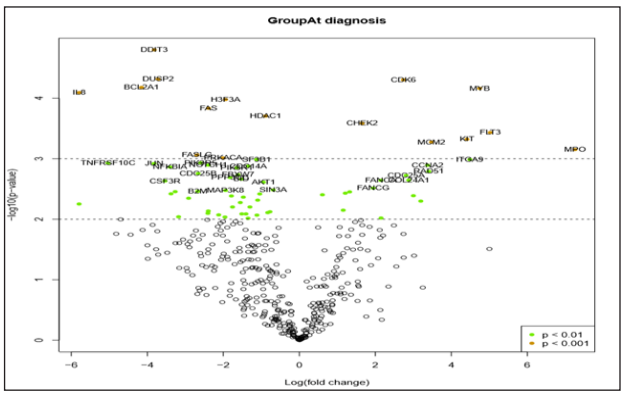

Fig. 2. Volcano plot of at diagnosis AML patients with normal cytogenetics.

This volcano plot for the group at diagnosis patients showed the most highlighted genes which lied on down regulated or up regulated genes according to the p-value. The green dots indicates p-value less than 0.01 whereas brown dots represents p-value less than 0.001. The log fold change increased as the plots scattered to the right and decreased as it scattered to the left.

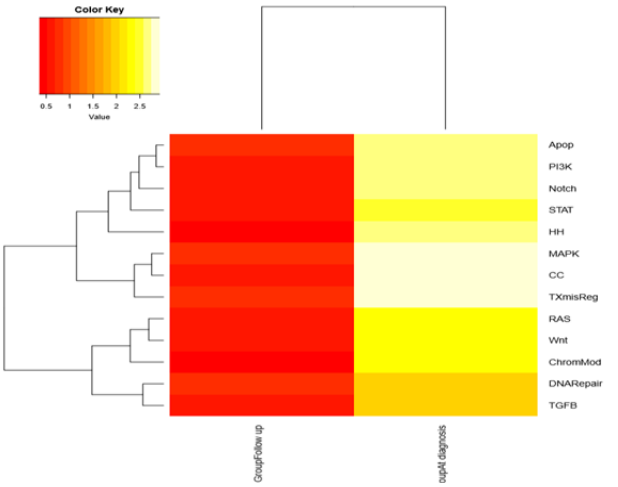

Fig. 3. The heat map of directed global significance scores shows that pathways Cell Cycle (CC)2, MAPK and TXmisReg are most enriched with differentially expressed genes in group at diagnosis patients.

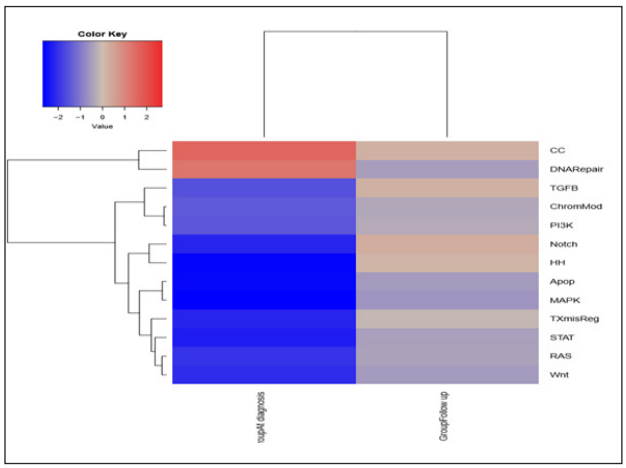

Fig. 4. High global significance statistics indicates extensive differential expression. Very high or low directed global significance statistics indicated extensive up or down regulation, respectively. Red indicated up regulated while blue indicated down regulated.

Cell cycle pathways contributed mostly in up regulated genes whereas MAPK and TXmisReg pathways involved on the most enriched down regulated genes as can be confirmed with global significance statistic highlighted in Figure 5.

Fig. 5. Global significance statistic for pathways vs. at diagnosis plots

The global significance statistic focused on measuring the cumulative evidence for the differential expression of genes in a pathway. Meanwhile, directed global significance statistic was alike to the global significance statistic, but rather than measuring the tendency of a pathway to have differentially-expressed genes, it also measured the tendency to have over or under expressed genes.

Figure 5 shows the highest-level view of cancer panel data. For the comparisons of pathways against at diagnosis patients, the global significance score of each pathway was plotted against the –log10(pathway score p-value) for its association with group difference. Cell Cycle, MAPK and TX mis Reg marked the most powerful deregulated under both metrics.

Discussion

NanoString requires smaller amounts of starting material, and can perform mRNA gene expression profiling with digital meticulousness. Therefore the results do not require further validation by another method. However, it is limited in detection to the screening panel available and may be more expensive when using greater numbers of samples[8].

The most highly expressed up regulated gene in patients at diagnosis in normal cytogenetic AML was Myeloperoxidase (MPO) gene which showed 7.25 logFC. MPO expression was significantly associated with disease-free survival (DFS). Previous report by Kim et al., (2012) demonstrated that the groups by the MPO expression in the intermediate cytogenetic risk group showed a significant difference in DFS (p<0.001). Meanwhile, fms-like tyrosine kinase 3 (FLT3) gene was spotted to be the secondly high up regulated after MPO gene with 5.02 logFC. A study by Valk et al., (2004) showed FLT3, a hematopoietic growth factor receptor, is the most common molecular abnormality in AML. The presence of such mutations in FLT3 and elevated expression of the transcription factor EVI1 convey a poor prognosis[9].

Therefore, N-MYC overexpression has also been testified in human acute myeloid leukaemia(AML), which we found out was a common event. N-MYC expression levels in AML samples from patients with favourable, intermediate, or unfavourable prognosis were compared with that in CD34+ cells from four healthy bone marrow donors[10]. According to Kawagoe et al., (2007), N-Myc overexpression in bone marrow powerfully endorses myeloid leukaemogenesis in mice and as its recurrent overexpression in human AML most likely contributed N-MYC to leukaemogenesis.

Meanwhile for the most highly down regulated gene in newly diagnosed AML goes to Granzyme B (GZMB) which scored -5.80 logFC. This gene were involved in cytolytic activity that showed high correlation with other transcripts expressed in activated cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells as well as lymphocyte activation-related gene validating it as a robust and specific metric of active cellular immunity[11].

IL-8 gene was found the second most down regulated gene in newly diagnosed patients with -5.79 logFC. Increased expression of IL-8 and/or its receptors has been characterized in cancer cells, endothelial cells, infiltrating neutrophils, and tumor-associated macrophages, suggesting that IL-8 may function as a significant regulatory factor within the tumor microenvironment[12]. IL-8 plays role in biological activities that includes regulation of some membrane molecules related to adhesion and migration process. Hence, its production by AML blasts might be importance for the pattern of leukaemic growth[13]. This goes oppositely with our findings where IL-8 found to be down regulated gene in normal cytogenetic AML at newly diagnosed patients. There is no recent study found to support our findings.

TNFRSF10C gene which is the third most down regulated gene that scored -5.03 logFC. It functioned as a tumor necrosis factor receptor superfamily, member 10c, which decoy without an intracellular domain and important in initiating apoptosis[14]. According to Pozzo, 2017, TNFRSF10C was involved in initiating apoptosis and myeloid development.

Conclusion

This descriptive findings highlighted the most enriched up regulated genes in newly diagnosed normal cytogenetic AML enlisted MPO, FLT3, MYCN, MYB, ITGA9, KIT, MCM2, RAD51, CCNA2 and

PROM1 which those commonly be found in AML cases. For highly expressed down regulated genes were GZMB, IL8, TNFRSFSF10C, LEF1, IL2RB, IL7R, BCL2A1, RASGRP1, IL1R2 and HSPA6. Thus, NanoString expression profiling may be a useful approach of exploring predictive of treatments, disease progression and its prognostic effects.

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Conflict of interest :

The authors declare that there is no conflict of interest

Authors contribution:

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