Research Article

Differential gene expression and co-regulated expression of genes in leukemia: an *in-silico* approach to identify potent biomarker

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Abstract

A biomarker can be measured, used to diagnose or classify disease, and measure progress as well as the therapeutic response of the disease. Early diagnosis and selection of appropriate treatment can be critical for the successful treatment of diseases. Identification and characterization of potent diagnostic biomarkers, and therapeutic targets rely heavily on traditional *in vitro* screens which require extensive resources and time. Integration of *in silico* screens prior to experimental validation can improve the efficiency and potency of biomarkers as well as reduce the cost and time of biomarker discovery. Considering the need, present work was undertaken to identify biomarkers for different classes of leukemia. Differential Gene Expression (DGE) analysis and co-regulated expression analysis were used for *in silico* identification and characterise a potent biomarker for leukemia. On the basis of *in silico* screening, the present study proposed seven protein-coding (CD38, TSC22D3, TNFRSF25, AGL, LARGE1, ARHGAP32, and PARM1) genes for the diagnosis of leukemia. The study also proposed a novel three-step lineage-specific model for the diagnosis of leukemia. In the three-step diagnosis model, the first group of biomarkers with an association of clinical and hematological parameters diagnose leukemia. The second group of biomarkers diagnoses acute and chronic form of leukemia. The third group of biomarkers identifies whether it belongs to myeloid lineage or lymphoid lineage.

Keywords: Biomarkers, Co-regulated Expression, Differential Gene Expression, Leukemia

INTRODUCTION

Leukaemia is a malignant neoplasm of hematopoietic cells. Aberrant gene expression related to cellular differentiation, and proliferation of hematopoietic cell contributes to malignant transformation of cells. A better understanding of differential gene expression (DGE) in leukemia should provide diagnostic biomarkers, and therapeutic targets for therapy of this disease. Differential gene expression analysis refers to the analysis, and interpretation of differences in the abundance of gene transcripts within a transcriptome (Conesa *et al*., 2016). DGE is important to understand the biological differences between healthy and diseased states (Rodriguez *et al*., 2017). Identification and characterization of potent diagnostic biomarkers, and therapeutic targets rely heavily on traditional *in vitro* screens which require extensive resources and time. Integration of *in silico* screens prior to experimental validation can improve the efficiency and potency of biomarkers as well as reduce the cost and time of biomarker discovery. Considering the need, present work was undertaken to

identify biomarkers for different classes of leukemia. In the present work, the combination of differential gene expression analysis and co-regulated expression analysis were used for *in silico* identification of potent biomarkers for leukemia.

MATERIALS AND METHODS

Microarray datasets selection

In order to find proper gene expression profiles in microarray datasets, a systematic search was performed in Gene Expression Omnibus (GEO) database (https://www.ncbi.nlm.nih.gov/geo/) using the keywords "AML" and "Serum", "CML" and "Serum", "Acute Lymphoblastic Leukemia" and "Serum". These broad searches yielded a large number of gene expression data sets. The search was first narrowed down to a few potential data sets using various filters such as "Homo sapiens" and "Expression Profile by Array" in the GEO database. Finally, by detailed study of data sets four normalized data sets were selected. The flowchart of the dataset selection procedure, filter features of data sets, and information of datasets are shown in Fig. 1.

### Selection and combination of top differentially expressed gene (DEG)

Differentially expressed genes of four different types of leukemia were analyzed using the online tool GEO2. GEO2R is an interactive tool that enables the analysis of GEO datasets. It uses a web-based program, that employs the Bioconductor packages, and limma R (Gentleman *et al*., 2004; Smyth, 2004). A Venn diagram was prepared for the combination of four different sets of four different types of leukemia. InteractiVeen (Heberle *et al*., 2015) online tool was used to combine all data set to find out overlapping and non-overlapping differentially expressed gene. In order to find the best common biomarker for all classes of leukemia, and to prevent missing critical genes, it was decided to select differentially expressed gene overlapped between at least two data sets.

Area under curve (AUC) analysis

The expression values of selected overlapping differentially expressed genes were extracted in the form of a soft file, and imported to GraphPad Prism software. Receiver-operating characteristic (ROC) curves, and area under the ROC curve (AUC) were used to assess the detection ability of each differentially expressed gene in the diagnosis of different classes of leukaemia from the control group based on the sensitivity and specificity of each differentially expressed gene. The higher the AUC, better the model would be.

Hierarchical clustering analysis

Individual expression values of significantly up/down-
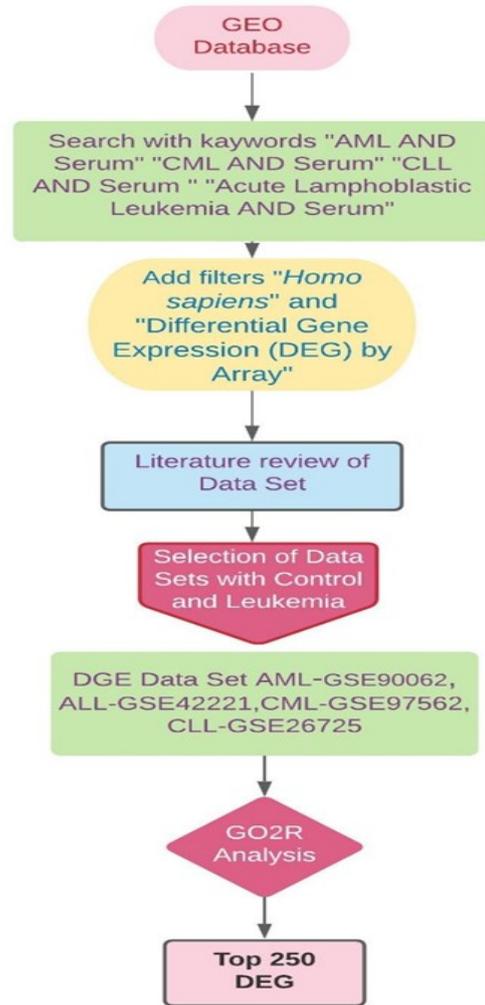
Fig. 1. *Microarray dataset selection procedure for GO2R Analysis, and identification of top 250 differentially expressed genes for four classes of leukemia.*

regulated differentially expressed genes in leukaemia were logarithm transformed, and were used as input values for the hierarchical clustering algorithm. The following criteria were applied: the distance chose "Pearson Correlation", and the linkage selected "average". The result is demonstrated as a Heatmap. Heatmapper webserver was used for the preparation of the heatmap.

RESULTS

Microarray dataset selection

The selected microarray dataset and their features for different classes of leukemia are shown in Table 1. The volcano plot and mean difference plot are presented from Fig. 2- 9 for each selected microarray dataset. A volcano plot displayed statistical significance (-log10 P value) versus magnitude of change (log2 fold chain) of differentially expressed gene and the mean difference plot displays log2 fold chain versus average log2 expression value. Highlighted genes were significantly

differentially expressed at a default adjusted p-value cut-off of 0.05 (red= upregulated and blue= downregulated).

Selection and combination of top differentially expressed gene (DEG)

After GEO2R analysis, 164 genes for AML, 202 genes for ALL, 146 genes for CLL and 194 for CML showed significantly up/downregulation. After integration of the results with the help of Venn diagram, one gene was common in AML, ALL and CLL, six genes were common in Acute leukemias (ALL and AML), five genes were common in Chronic leukemias (CML and CLL), four genes were common in Myeloid leukemias (AML and CML) and six genes were common in Lymphoid leukemias (ALL and CLL). Details of common genes are shown in Fig. 10 and Table 2.

Area under curve (AUC) analysis

On the basis of the lowest adjusted p value of common differentially expressed seven genes from different groups (CD38, TSC22D3, TNFRSF25, AGL, LARGE1, ARHGHP32, and PARM1) were selected for further analysis. After extraction of expression values for all seven DEGs, in order to find the reliable marker in discriminating leukemia from healthy controls, AUC analysis was performed and ROC curves for seven DEG were prepared. ROC curve AUC values of seven DEG for different groups are shown in Table 3.

Hierarchical clustering analysis and co-expression correlation

Fig. 11-14 demonstrates hierarchically clustered Heatmaps built up using the expression values of DEGs for four classes of leukaemia. Four differentially expressed genes AGL, CD38, TNFRSF25, TSC22D3 are shown in the Heatmap of AML. AGL and CD38 showed downregulation and TNFRSF25, TSC22D3 showed upregulation in AML patients. Similarly, four differentially expressed genes LARGE1, TNFRSF25, CD38, TSC22D3 are shown in the Heatmap of ALL. LARGE1, TNFRSF25, and TSC22D3 showed co-expression and upregulation in ALL patients and CD38 showed down-regulation. Three differentially expressed genes AGL, ARHGAP32, and PARM1 are shown in the Heatmap of CML. ARHGAP32 and PARM1 showed downregulation and AGL showed upregulation in CML patients. Four differentially expressed genes LARGE1, ARHGAP32, CD38, and PARM1 are shown in the Heatmap of CLL. All four genes showed similar co-expression patterns and down-regulated in CLL.

DISCUSSION

In recent years, there has been an increased emphasis on personalized and targeted therapy for leukemia patients. Therefore, it is important to explore the mechanism for the development of novel therapeutic strategies. Recently, differential gene expression profiling analysis has been widely used to reveal abnormal gene expression patterns related to leukemia, to identifying novel diagnostic and therapeutic targets. In the present study, differential gene expression analysis and co-regulated gene expression analysis were used to identify some novel diagnostic and therapeutic targets.

By using *in silico* methods, the present study proposed seven new protein-coding genes for the diagnosis of

Table 1. Selected microarray dataset for GO2R analysis, and identification of top 250 differentially expressed genes for four classes of leukemia.

| S.N.	GEO accession number	Leukemia	Platform	Samples	Authors
01	GSE90062	AML	GPL15207	Control=03, AML=03	Li *et al.,* 2017
02	GSE42221	ALL	GPL96	Control=04, ALL=07	Harder *et al.,* 2013
03	GSE97562	CML	GPL6244	Control=20, CML=20	Aviles *et al.,* 2017
04	GSE26725	CLL	GPL570	Control=05, CLL=12	Vargova *et al.,* 2011

Table 2. Details of Common Differentially Expressed Gene (DEG).

| Leukemia	Number of common protein coding DEG	Symbol of common protein coding DEG
AML, ALL, CLL	1	CD38
ALL, AML	6	GJA1, SH3BP5, SLC1A4, TSC22D3, PHGDH, TNFRSF25
CLL, CML	5	ARHGAP32, FRY, CAMK1D PIK3R6, PARM1
AML, CML	4	AGL, LHFP, CHCHD7, TMEM154
ALL, CLL	6	ANP32E, LARGE1, IGLC1, CYAT1, SMAGP, ITM2C

Table 3. AUC analysis for seven DEGs.

Leukemia type	Gene	Area	Stander error	p value
AML	CD38	0.8762	0.0524	0.0001
AML	TSC22D3	1.0000	0.0000	0.0495
AML	TNFRSF25	1.0000	0.0000	0.0495
AML	AGL	1.0000	0.0000	0.0490
ALL	CD38	1.0000	0.0000	0.0082
ALL	TSC22D3	1.0000	0.0000	0.0082
ALL	TNFRSF25	1.0000	0.0000	0.0495
ALL	LARGE1	1.0000	0.0082	0.0082
CML	ARHGAP32	0.8800	0.0590	0.0001
CML	PARM1	0.8825	0.0549	0.0001
CML	AGL	0.08513	0.0598	0.0001
CLL	CD38	1.0000	0.0000	0.0016
CLL	ARHGAP32	0.9236	0.07371	0.0004
CLL	PARM1	1.0000	0.0000	0.0016
CLL	LARGE1	1.0000	0.0000	0.0016
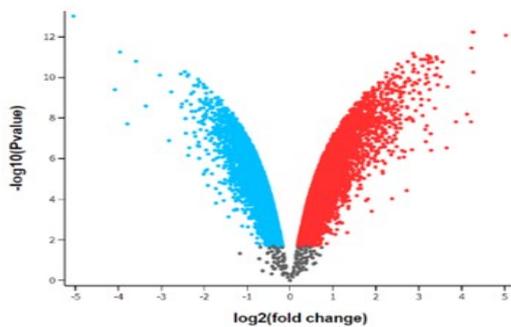
Fig. 2. *Volcano plot of AML.*
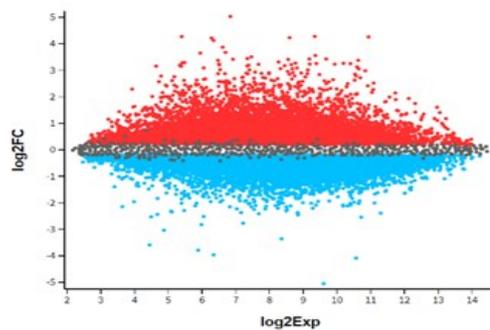
Fig. 3. *Mean difference plot of AML*

Fig. 4. *Volcano plot of ALL.*

Fig. 5. *Mean difference plot of ALL.*
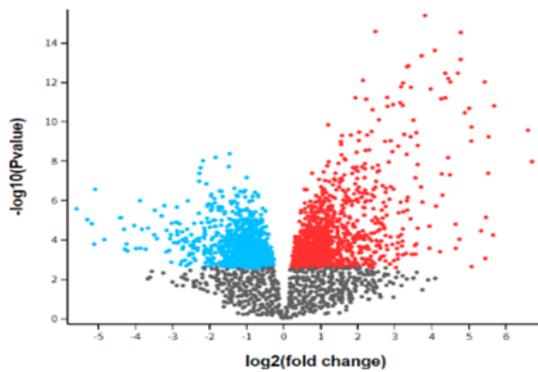
Fig. 6. *Volcano plot of CLL.*
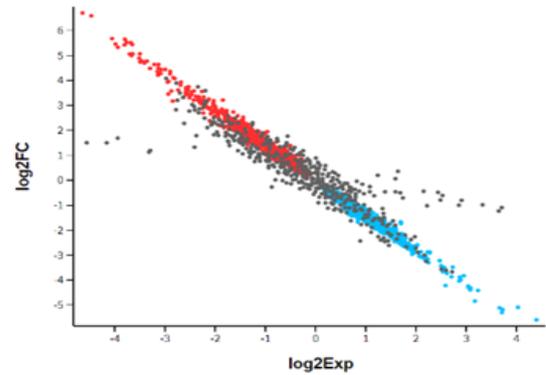
Fig. 7. *Mean difference plot of CLL.*

Fig. 8. *Volcano plot of CML.*

Fig. 9. *Mean difference plot of CML.*

leukemia. To identify protein-coding genes as bi-omarkers, four GEO microarray datasets containing the expression profiles from the serum of leukemia patients and healthy controls were chosen for differential expression analysis. Four GEO microarray dataset GSE90062, GSE42221, GSE97562, and GSE26725 comprises gene expression data for AML, ALL, CML, and CLL respectively. The gene expression profile data were downloaded, and analyzed using the GEO2R. Top 250 genes were selected from each microarray data sets. Common genes between different classes of leukemia were captured out with the help of the Venn diagram. On the basis of the lowest adjusted p values, seven genes were selected from different groups of leukemia. The expression values for all these differentially expressed genes were extracted and normalized. Using Area Under ROC curve Analysis (AUC), the most powerful DEGs in discriminating the leukemia patient from a healthy control were picked out. In the present study, a total seven genes showed AUC > 0.800 and considered for further analysis. The co-expression pat-

tern of seven identified genes was analyzed by hierarchically clustered Heatmaps built up using the expression values of DEGs. The present study also proposed a three-step lineage-specific model for the diagnosis of leukemia. In the three-step diagnosis model, the first group of biomarkers with an association of clinical and hematological parameters diagnose leukemia, the second group of biomarkers diagnoses acute and chronic form of leukemia and the third group of biomarkers identifies whether it belongs to myeloid lineage or lymphoid lineage.
Tripathi and Pandya (2016) carried out a similar study when they studied differential analysis of gene expression for acute myeloid leukemia. They analyzed the transcriptional profiling of human T-cell involved in the AML disease and reported CD4, NA, ADTRP genes as a diagnostic and therapeutic biomarker. Tang *et al*. (2019) reported the five key genes: ACTR2, ARPC3, ARPC5, CTTN, and APP may be implicated in tumor progression and could potentially represent promising prognostic biomarkers and therapeutic targets for AML
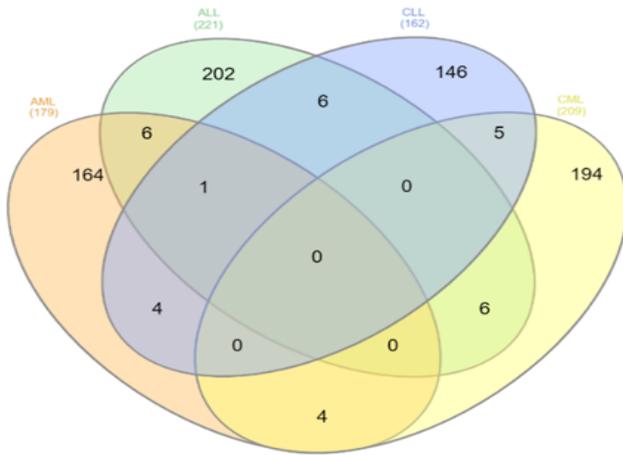
Fig. 10. *Venn diagram of common differentially expressed gene (DEG).*

patients. Wei Fu *et al*. (2020) reported abnormal expression of CYBB, CYFIP2, SERPINE1, and ITGAM based on high-throughput data analysis. Sanchez and Mackenzie (2020) reported integrative network analysis of differentially methylated and expressed genes for

biomarker identification in leukemia. There are many studies that have been reported (Prada *et al.,* 2017, Jiang *et al.,* 2016, Sweet *et al.,* 2013) for the identification and characterization of biomarkers for individual classes of leukemia, but the present study was carried out for the comparative analysis of biomarker profiling of major classes (AML, ALL, CML, and CLL) of leukemia.

In the present study, the CD38 gene is a common differentially expressed gene in AML, ALL, and CLL. AUC analysis of CD38 shows, it is a good classifier marker for ALL and CLL. Jiang *et al*., (2016) also reported CD38 expression in HSCs, Leukemic stem cells, and B-ALL.

There are six differentially expressed genes that are commonly involved in acute leukemias. Out of these TSC22D3 and TNFRSF25 were selected on the basis of the lowest p-value. AUC analysis of TNFRSF25 shows that, it is a good classifier marker for acute leukemia. Miller *et al*., (2007) reported TSC22D3 (TSC22 domain family member) was highly induced in all the leukemic cells tested, regardless of apoptotic sensitivity
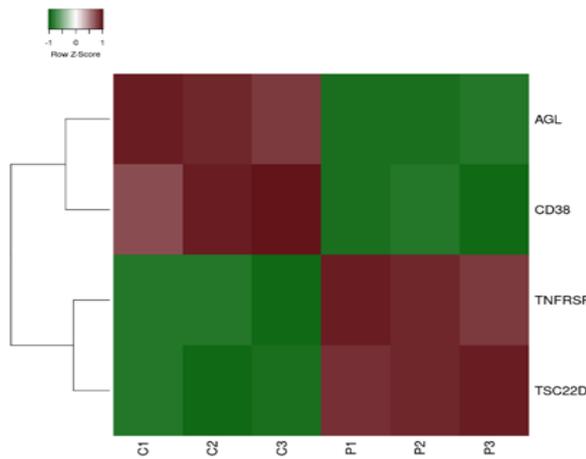
Fig. 11. *Heatmap for AML.*
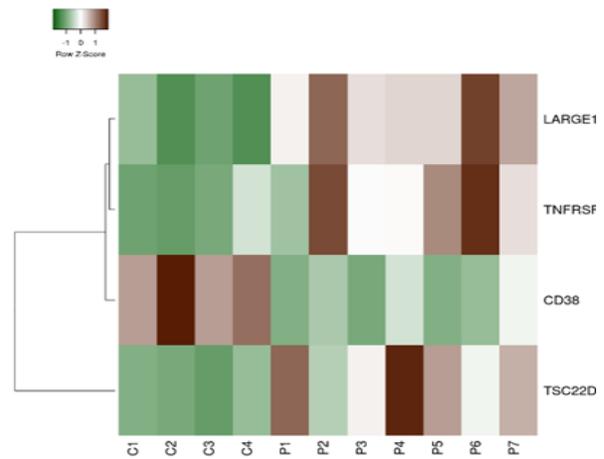
Fig. *12 Heatmap for ALL.*
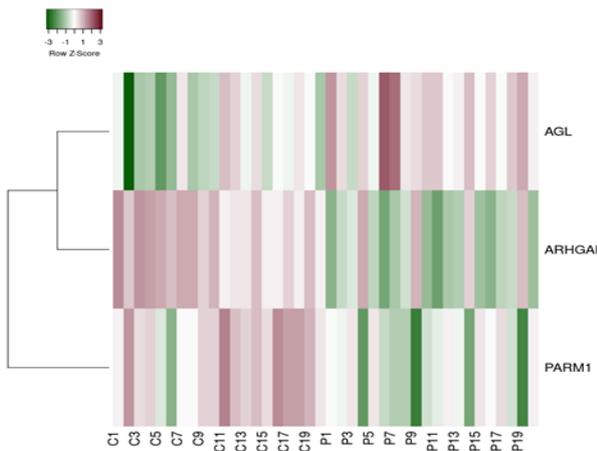
Fig. 13. *Heatmap for CML.*
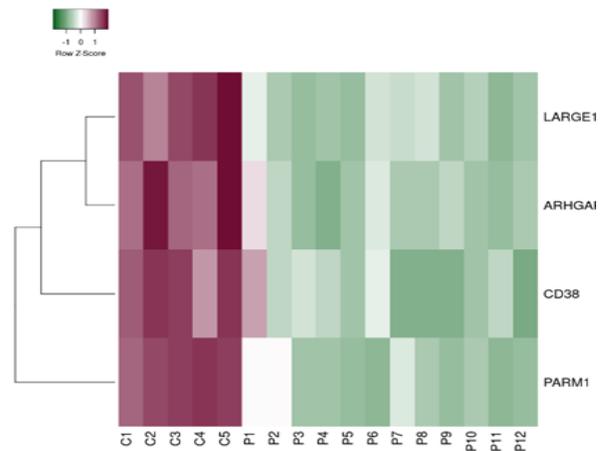
Fig. 14. *Heatmap for CLL.*

or resistance. Yang *et al.* (2019) reported the role of upregulated TSC22D3 and stress-induced glucocorticoid surge in therapy-induced anticancer immunosurveillance. Paul *et al.*, (2008) studied that TNFRSF25 (Tumor necrosis factor receptor superfamily member 25) are hypermethylated in tumor tissue. TNFRSF25 was significantly up-regulated in ECFCs (Endothelial colony-forming cells) from VTE (Venous thromboembolism) patients. Through experiments of functional validation, they further demonstrated that the upregulation of TNFSF15–TNFRSF25 axis sustains reduced survival and proliferation of ECFCs in VTE patients, thus suggesting that this molecular pathway, by impairing endothelial repair, may contribute to VTE pathogenesis.

Five genes were differentially expressed in chronic leukemia. Out of these ARHGAP32 and PARM1 were selected on the basis of the lowest p-value. AUC analysis of these two genes shows both are good classifier markers for CLL. A similar observation was reported by Vlaanderen *et al.* (2017), in which they reported ARHGAP32 as a pre-diagnostic blood transcriptome marker of chronic lymphocytic (CLL). Paulisally *et al.*, (2014) identified PAMR1 (Peptidase domain-containing associated with muscle regeneration 1) as being frequently suppressed in breast cancer tissues. They reported that PAMR1 expression was reduced in all tested breast cancer cell lines, while PAMR1 was expressed moderately in normal breast tissues and primary mammary epithelial cells. Li (2020), reported the PARM1 key target gene for mir-382-5P regulating ovarian cancer.

In the present study, four common genes are differentially expressed in myeloid leukemia and six genes in lymphoid leukemia. AUC analysis of these genes showed AGL and TMEM154 is good classifier marker for AML and LARG1 is good classifier marker for both lymphoid leukemia. In the present study, the overall expression of AML (Fig. 2 and 3 Volcano plot and mean difference plot) showed that more differentially expressed genes were overexpressed. 164 genes were exclusively expressed in AML. Heatmap of CD38 showed that it is an overexpressed gene in AML and it is downregulated in ALL and CLL. In addition to CD38, AGL and TMEM154 are good classifier marker for AML. Crushell *et al.*, (2010) confirmed that AGL encodes the glycogen debrancher enzyme, which is involved in glycogen degradation, and it was significantly altered, carbohydrate metabolic process in response to glucocorticoid stimulus. Guin *et al.*, (2016) confirmed that AGL also involved in the induction of hyaluronic acid synthesis and further regulate tumor growth in bladder cancer. Holroyde *et al.*, (1984) showed that diverse abnormalities of the carbohydrate metabolic process often occurred in cancer cachexia, such as colorectal cancer. In addition, aerobic glycolysis was ensured to be a metabolic adaptation that promotes the proliferation of colorectal cancer cells (Straus *et al.*, 2013).

In the present study, the overall expression of ALL (Fig. 4 and 5 Volcano plot and mean difference plot) showed that more differentially expressed genes were underexpressed. 202 genes were exclusively expressed in ALL. In contrast with AML, the heatmap of CD38 showed that CD38 was an under-expressed gene in ALL. AUC analysis showed ARHGAP32, PARM1, and LARGE1 can be used as biomarkers for ALL. The overall expression of CLL and CML (Fig. 6- 9 Volcano plot and mean difference plot) showed that more differentially expressed genes were overexpressed. 146 genes were exclusively expressed in CLL and 194 genes were exclusively expressed in CML.

Conclusion

Using multiple bioinformatics tools and on the basis of the lowest adjusted p value and AUC analysis present study proposed seven differentially expressed genes (CD38, TSC22D3, TNFRSF25, AGL, LARGE1, ARHGAP32, and PARM1) as biomarkers for leukemia. In conclusion, this study supports the accuracy of some formerly proposed biomarkers (CD38, and AGL) for leukemia, and also has suggested new candidate biomarkers (TSC22D3, TNFRSF25, LARGE1, ARHGAP32, and PARM1) which can be used as diagnostic or prognostic means or as therapeutic targets.

Conflict of interest
The authors declare that they have no conflict of interest.

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