Two-dimensional electrophoresis protein profiles of HL-60 and CCRF-CEM cell lines treated with epigenetic modification drugs

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Abstract. Leukemia is classified as a malignant disease of hematopoietic stem cells (HSCs) that fails in cell differentiation but preserve their self-renewal. It is caused by genetic alterations and epigenetic modifications resulting in the activation or inactivation of particular genes for transcription. Epigenetic causes changes in gene expression without any alteration in the DNA sequence. The most common epigenetic modifications are DNA methylation and histone acetylation. 5-Azacytidine (5-Aza) is a DNA methyltransferase inhibitor (DNMTi) that inhibits DNA methyltransferase enzymes resulting in hypomethylation. Trichostatin A (TSA) is a histone deacetylase inhibitor which inhibits deacetylation of both histone and non-histone proteins resulting in chromatin relaxation. This present study focused on the alteration of proteome profile on 2D gel electrophoresis (2-DE) induced by 5-Aza and TSA in HL-60 and CCRF-CEM cell lines as *in vitro* model to represent acute promyelocytic leukemia (APL) and T-lymphoblastic leukemia (T-ALL), respectively. Total proteins of untreated and 5-Aza/TSA-treated HL-60 and CCRF-CEM cell lines were extracted using urea/thiourea buffer and stained with Coomassie Blue. Comparative analysis of untreated and 5-Aza/TSA-treated HL-60 and CCRF-CEM was performed by PDQuest software. Qualitative analysis identified 190-659 protein spots detected in untreated, 5-Aza and TSA-treated HL-60 and CCRF-CEM. Quantitative comparison analysis was analyzed by over 2-fold change in 5-Aza/TSA-treated cells compared to untreated. One and eight upregulated proteins were detected in 5-Aza and TSA-treated HL-60, respectively. While five and one upregulated proteins were detected in 5-Aza and TSA-treated CCRF-CEM, respectively. These preliminary results suggested that 5-Aza and TSA induced proteome profiles alterations due to their inhibition effects in HL-60 and CCRF-CEM cell lines.

Keywords: Epigenetic, 2-DE, 5-Aza, TSA, HL-60, CCRF-CEM

INTRODUCTION

Leukemia is a type of blood cancer due to the accumulation of malignant hematopoietic stem cells (HSCs) that is lost in cell differentiation but

preserve their self-renewal result in bone marrow failure and tissue infiltration (Luczak *et al.*, 2012b; Qureshi and Hall, 2013). Leukemia arises from

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lymphocytes, myeloid cells, erythrocytes, and others in the bone marrow, lymph nodes or spleen. Regardless of the cell type origin, the course of leukemia is either acute; aggressively progressing or chronic; slowly progressing (Ntziachristos *et al.*, 2013).

Acute promyelocytic leukemia (APL) is a distinct subtype of acute myeloid leukemia (AML), categorized as APL with t(15;17)(q22;q11-12), promyelocytic leukemia-retinoic acid receptor alpha (PML-RARA) by World Health Organization (WHO) and AML-M3 in French-American-British (FAB) classification system (Estey, 2015; Karim *et al.*, 2014). It is categorized by abnormal overgrowth of immature granulocytes (promyelocytes) (Wang *et al.*, 2014). Previous study reported that the annual incidence rate of APL was 0.08 cases per 100 000 population and 5-8% of AML cases in younger patients (Arber *et al.*, 2017). Coagulopathy occurs in most APL patients and it is life-threatening (Breen *et al.*, 2012).

T-lymphoblastic leukemia (T-ALL) is a neoplasm of T-lymphocytes (lymphoblasts) by WHO. T-ALL presents as a tissue-based mass of thymus, swollen lymph nodes or extranodal sites (You *et al.*, 2015). T-ALL patients have abnormal karyotype and the most frequent cytogenetic abnormality are translocations of alpha and delta T-cell receptor loci at 14q11.2, the beta locus at 7q35, or the gamma locus at 7p14-15 (Taylor and Wang, 2012). Previous study reported that T-ALL comprises of 15% among childhood acute lymphoblastic leukemia (ALL) and 25% among adult ALL cases (Borowitz *et al.*, 2017).

Genetic alterations such as deletion, mutations or chromosomal translocations lead to leukemia. Epigenetic is another molecular mechanism that also promotes leukemia initiation by silencing tumor suppressor genes (Ahuja *et al.*, 2016). Epigenetic causes changes in gene expression that do not change the DNA sequence (Stahl *et al.*, 2016). The most common epigenetic modifications involve DNA methylation and histone acetylation (Deng *et al.*, 2016; Matoušová *et al.*, 2014). DNA methylation is an addition of methyl group to the 5-carbon position of cytosine by DNA methyltransferases (DNMTs) resulting in 5-methylcytosine (Wouters and Delwel, 2016). Histone acetylation involves the transfer of acetyl to lysine residues in histone proteins. Histone

acetyltransferase (HAT) transfers acetyl whereas histone deacetylase (HDAC) removes acetyl from histone (West and Johnstone, 2014).

Hypermethylation due to aberrant DNA methylation of promoter region resulting in silencing of tumor suppressor genes and transcription inactivation have been shown in all stages of cancer (Huang *et al.*, 2011; Matoušová *et al.*, 2014). Unbalanced activity of HAT and HDAC such as over expression of HDAC lead to chromatin condensation and inactivation of tumor-suppressor genes in cancer cells (Deng *et al.*, 2016). Unlike genetic alterations, epigenetic is reversible and the modifications of affected genes could be recovered and re-programmed to normal state (Ahuja *et al.*, 2016).

5-Aza was first synthesized almost 40 years ago by Piskala and Sorm (Christman, 2002). 5-Aza inhibits DNMT1, DNMT3A and DNMT3B to result in DNA hypomethylation and re-activation of silencing tumor suppressor genes (Wongtrakoon, 2015). 5-Aza has been approved for the treatment of myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML) with low blast count by the US Food and Drug Administration (FDA) (Wouters and Delwel, 2016). 5-Aza showed positive effect by re-expressing *Gata2* in *Tet2*-null mice with *FLT3-ITD* mutation to produce cell differentiation (Shih *et al.*, 2015).

TSA, a hydroxamate acid-based pan-HDACi, previously identified as an anti-fungal agent derived from metabolites of strains *Streptomyces hygroscopicus* (Tsuji *et al.*, 1976), was used in clinical trials to inhibit HDACs class I and II for hematological malignancies (Spiegel *et al.*, 2012). TSA inhibits HDAC activity and increases accumulation of histone acetylation by releasing acetic acid from acetylated histone (Bantscheff *et al.*, 2011). Previous studies reported that TSA induced less HDAC9 expression in AML blasts and cell lines (Bradbury *et al.*, 2005). The epigenetic modification drugs such as 5-Aza and TSA specifically target to reactivate the silencing of tumor suppressor genes resulted in cell differentiation and apoptosis (Fard *et al.*, 2012; Hollenbach *et al.*, 2010; Horing *et al.*, 2013; Yu *et al.*, 2008).

The main focus of proteomic study is the proteome, a set of proteins occurring at a given time and a given place in an organism. Proteomic

analysis creates possibility to determine how various diseases affect the pattern of protein accumulation and contribute to the development of treatment strategies (Luczak *et al.*, 2012a). Proteomic analysis provides a detailed overview of differentially expressed proteins in cellular pathways and disease processes (Almaiman *et al.*, 2016). The differentially expressed proteins provide information on the biochemical changes that may explain the mechanism underlying leukemia progression.

2D gel electrophoresis (2-DE) is a technique to separate complex protein mixtures based on their isoelectric point (first dimension) and molecular weight (second dimension) (Almaiman *et al.*, 2016). Coupled with Coomassie blue staining and image analysis, 2-DE allows for the simultaneous detection and relative quantification of several hundreds of individual proteins (Pallen *et al.*, 2014). It is widely used to study qualitative and quantitative protein changes between different states of a cell (e.g. healthy and diseased), conditionally expressed protein and posttranslational modifications. This technique has its ability for comparative proteomic analysis to screen biomarkers and identification of drug targets (Kumar *et al.*, 2017).

Therefore, in the present study, HL-60 and CCRF-CEM leukemic cell lines representing APL and T-ALL were utilized to evaluate 2-DE proteome profiles alteration between untreated and 5-Aza/TSA-treated environment. The evaluation was performed based on all detectable protein spots corresponding to the different cell groups. Two types of analyzes were conducted: qualitative analysis, referring to the presence or absence of protein spots and quantitative analysis, referring to the optical intensity of protein spots.

MATERIALS AND METHODS

The culture and treatment of cell lines. CCRF-CEM (human T-lymphoblastic leukemia), donated by Integrated Centre for Research Animal Care and Use (ICRACU), International Islamic University Malaysia and HL-60 (human acute promyelocytic leukemia) donated by Faculty of Health Sciences, University of Sultan Zainal Abidin were originally purchased from American

Type Cell Culture (ATCC) (MD, USA). The cell lines were cultured in Roswell Park Memorial Institute (RPMI)-1640 (Gibco, CA, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, CA, USA) and 1% (v/v) penicillin-streptomycin (Invitrogen, CA, USA) at 37°C in a humidified atmosphere air with 5% CO₂. The IC₅₀ of 5-Aza and TSA in HL-60 and CCRF-CEM were established previously (Azizee *et al.*, 2018). HL-60 was treated with 5-Aza at IC₅₀ (2.01 µM) and TSA at IC₅₀ (2.35 µM) for 72 and 48 hours, respectively. CCRF-CEM was treated with 5-Aza at IC₅₀ (1.98 µM) and TSA at IC₅₀ (2.65 µM) for 72 hours.

Total protein preparation. The total protein of untreated and 5-Aza/TSA-treated HL-60 and CCRF-CEM (10-30 x 10⁶ cells/ml) were homogenized with urea/thiourea buffer (7M urea, 2M thiourea, 1% ASB-14, 40mM Tris, 0.001% Bromophenol Blue 200mM Tributylphospine (TBP) and 0.2% ampholyte) supplemented with protease inhibitor. Cells were placed on ice and sonicated with an ultrasonic probe (Daihan Scientific Co, Gangwon-do, KR) to disrupt cells and fragment the genomic DNA. The samples were sonicated for 3-4 times or until lysis was completed. The samples were chilled on the ice briefly between each ultrasonic treatment and centrifuged at 16,000 x g for 30 mins at 18°C. The supernatant was transferred to a 1.5 ml centrifuge tube (Eppendorf, NY, USA) and the protein concentration for each cell extract was measured by RC DC protein assay (Bio-Rad Laboratories, CA, USA). The cell extracts were stored at -80°C (Thermos Fisher scientific, MA, USA) for 2-DE.

2D gel electrophoresis (2-DE). Prior to isoelectric focusing (IEF), 600 µg of total protein was dissolved with 1% of ampholyte (Bio-Rad Laboratories, CA, USA) and rehydrated in passive condition using 7 cm IPG strip pH 4-7 (Bio-Rad Laboratories, CA, USA). The PROTEAN il2 IEF (Bio-Rad Laboratories, CA, USA) was programmed under 250 V for 20 mins, 4000 V for 2 hours, 4000 V for 14000 Volt-hours and 500 V for stabilizing to run IPG strip. The IPG strip was equilibrated with equilibration buffer I (0.06 g of 6M urea, 0.625 mL of 1.5M Tris-HCl, 0.25 mL of 20% (v/v) SDS solution, 1 mL of 20% glycerol, 0.6 mL of distilled water and 0.025 mL of 1%

(w/v) dithiothreitol) for 10 mins and equilibration buffer II (0.06 g of 6M urea, 0.625 mL of 1.5M Tris-HCl, 0.25 mL of 20% (v/v) SDS solution, 1 mL of 20% (v/v) glycerol, 0.6 mL of distilled water and 0.025 mL of 2% (v/v) iodoacetamide) for 10 mins at room temperature with gentle shaking. Electrophoresis was run at a constant volt of 90 V for 1 hour and 50 mins in a Mini-PROTEAN tetra cell (Bio-Rad Laboratories, CA, USA). After gel electrophoresis completed, the gel was stained with Coomassie blue (Bio-Rad Laboratories, CA, USA) for one hour.

Image analysis. The gel image was captured using GS-800 Calibrated densitometer (Bio-Rad Laboratories, CA, USA). Scanned 2-DE gel images were analyzed with PDQuest software (Bio-Rad Laboratories, CA, USA). The spots detection was based on spots intensities. Briefly, any artifacts (speckles) on 2-DE gel images were removed by setting the minimum spot diameter to a level greater than the diameter of the speckles. The process of detecting spots was automated and required significant input parameters (spot size; light, small and large) in order to register the spots correctly. PDQuest software applied these parameters to detect majority of protein spots on 2-DE gel image and several protein spots were

manually defined (e.g. adding or removing spots). A match set was created for comparison analysis to match the corresponding spots across gels according to the master gel (selected based in good spot quality). Automatic matching was performed to highlight similarities and differences for quantitative comparison. Each 2-DE gel image was normalized to reduce variability in terms of protein load per gel, staining efficiency or image capture. All comparative analyses were statistically analyzed.

RESULTS AND DISCUSSION

2-DE proteome profiles of HL-60 and CCRF-CEM. Figures 1 and 2 showed representative 2-DE protein profiles of untreated, 5-Aza- and TSA-treated HL-60 and CCRF-CEM. Table 1 and 2 demonstrated qualitative analysis of protein spots detected in each gel of untreated, 5-Aza- and TSA-treated HL-60 and CCRF-CEM. 2-DE gel images were analyzed in duplicate to minimize gel-to-gel variation. Protein spots were reproducible as both cell groups have a close matching rate.

Table 1. Qualitative analyzes of protein spots detected of untreated, 5-Aza and TSA-treated HL-60.

HL-60		Gel 1	Gel 2	Gel 3	Reproducible spots	Matching rate ^a (%)	Correlation coefficient
Untreated	Extract 1	483	545	501	117	69	0.628
	Extract 2	519	562	521		72	0.632
5-Aza-treated	Extract 1	620	642	601	392	89	0.729
	Extract 2	557	659	613		79	0.729
TSA-treated	Extract 1	352	350	349	190	86	0.626
	Extract 2	490	468	505		77	0.774

^aMatching rate represents the percentage of matched spots on the gel relative to the total number of spots on the master gel.

Table 2. Qualitative analyzes of protein spots detected of untreated, 5-Aza and TSA-treated CCRF-CEM.

CCRF-CEM		Gel 1	Gel 2	Gel 3	Reproducible spots	Matching rate ^a (%)	Correlation coefficient
Untreated	Extract 1	257	217	318	231	77	0.727
	Extract 2	319	414	385		75	0.759
5-Aza-treated	Extract 1	190	359	305	271	64	0.727
	Extract 2	378	185	255		60	0.773
TSA-treated	Extract 1	377	194	410	105	48	0.625
	Extract 2	347	334	348		61	0.728

^aMatching rate represents the percentage of matched spots on the gel relative to the total number of spots on the master gel.

Figure 1. 2-DE gel images of protein from HL-60. (A) Untreated, (B) 5-Aza-treated, and (C) TSA-treated. SDS = 2nd Dimension Separation, MW = Molecular weight.

Comparative 2-DE proteome profiles between untreated and treated HL-60.

Quantitative comparison of 2-DE protein profiles between untreated and 5-Aza/TSA-treated HL-60 and CCRF-CEM were analyzed to detect differential expressed proteins. Corresponding protein spots were identified (labelled by SSP) based on intensities whose volume changed across different groups (i.e. untreated and 5-Aza/TSA-treated). Upregulated proteins were represented with high spot intensity in 5-Aza/TSA-treated but low spot intensity in untreated 2-DE gel images, and vice versa for downregulated proteins. Differential expressed proteins were manually checked to confirm their presence or absence in each individual 2-DE gel images.

Figure 2. 2-DE gel images of protein from CCRF-CEM (A) Untreated, (B) 5-Aza-treated and (C) TSA-treated. SDS=2nd Dimension Separation, MW=Molecular weight.

Table 3 demonstrated quantitative analysis of proteome profiles between untreated and 5-Aza/TSA-treated HL-60 by 2-fold change. The comparative analyses demonstrated 76 of differential expressed proteins were detected between untreated and 5-Aza-treated HL-60 made up of 45 upregulated and 31 downregulated protein spots. Whereas, 95 of differential expressed proteins were detected between untreated and TSA-treated HL-60 made up of 25 upregulated and 70 downregulated protein spots. Figures 3 and 4 showed representatives of 2-DE images of upregulated and downregulated proteins between untreated and 5-Aza-/TSA-treated HL-60. Correlation of variation (CV) of $\geq 70\%$ identified 1 and 8 upregulated protein spots between untreated and 5-Aza/TSA-treated HL-60 (Tables 4 and 5, respectively).

Table 3. Quantitative analysis of protein spots detected, spots matched, matching rate and mean coefficient of variation in untreated, 5-Aza- and TSA-treated HL-60.

Sample group	No. of protein spots detected	No. of protein spots matched	Matching rate ^b (%)	Mean coefficient of variation (CV)
Untreated 1	506	195	30	72.20
Untreated 2	542	162	26	
Untreated 3	514	151	24	
5-Aza-treated 1 ^a	620	620	100	51.47
5-Aza-treated 2	642	163	26	
5-Aza-treated 3	603	153	25	
TSA-treated 1 ^a	490	490	100	75.47
TSA-treated 2	468	74	14	
TSA-treated 3	505	83	16	

^a5-Aza-treated 1 and TSA-treated 1 denoted as a master gel (good protein spots intensity) by PDQuest software.

^bMatching rate represents the relativeness of protein spots on a particular gel correspond spots on the master gel. Independent *t*-test, ($p < 0.05$).

Figure 3. Representative of 2-DE gel images: (A) Upregulated protein spots (i), 5-Aza-treated and (ii), untreated HL-60. (B) Downregulated protein spots (i), 5-Aza-treated and (ii) untreated HL-60. X dots denote the upregulated or downregulated protein spot label with standard spot number (SSP). The same SSP represents the same protein on different gels (Results from PDQuest 2-D analysis software).

Figure 4. Representative of 2-DE gel images: (A) Upregulated protein spots (i), TSA-treated and (ii), untreated HL-60. (B) Downregulated protein spots (i), TSA-treated and (ii) untreated HL-60. X dots denote the upregulated or downregulated protein spot label with standard spot number (SSP). The same SSP represents the same protein on different gels. (Results from PDQuest 2-D analysis software).

Table 4. List of differential expressed proteins identified by quantitative analysis between untreated and 5-Aza-treated HL-60.

	SSP ^a	MW ^b (kDa)	pH ^c	Spot intensity (OD ^d)		Fold difference (Relative to Untreated)	CV ^e (%)
				Untreated	5-Aza- treated		
Up regulated	3804	86.0	5.8	896.2	974.4	1.1	93.1
	5701	77.4	5.6	19141.2	25401.2	1.3	77.8
	6901	123.9	5.3	101.4	520.7	5.14*	75.7
Down regulated	5108	34.3	5.5	27548.5	2067.3	0.1	70.9
	2603	67.7	6.2	5825.5	850.1	0.2	90.4

^aSSP - standard spot number denotes the same protein spot on different gels.

^bMW - theoretical molecular weight (kDa) obtained from PDQuest software.

^cpI - theoretical isoelectric charge (pH) obtained from PDQuest software.

^dOD - optical density measured from the densitometer.

^eCV - correlation of variation must be $\geq 70\%$; indicates how close of individual gel image match within the replicate group with the same sample and gel electrophoresis.

* ≥ 2 -fold change.

Table 5. List of differential expressed proteins identified by quantitative analysis between untreated and TSA-treated HL-60.

	SSP ^a	MW ^b (kDa)	pH ^c	Spot intensity (OD ^d)		Fold difference (Relative to Untreated)	CV ^e (%)
				Untreated	TSA- treated		
Up regulated	5603	87.7	5.1	14203.3	14970.6	1.1	86.8
	2704	92.6	6.1	3499.6	4509.0	1.3	77.6
	5102	28.9	5.1	232.6	386.0	1.7	75.7
	4903	151.9	5.6	222.3	556.4	2.5*	87.0
	5801	141.5	5.2	446.2	1396.9	3.1*	84.3
	2702	93.3	6.2	1373.9	5859.7	4.3*	76.9
	4803	134.6	5.6	571.5	2636.0	4.6*	91.5
	5804	131.5	5.0	2602.7	12115.4	4.7*	85.9
	4302	36.8	5.8	162.2	1086.1	6.7*	88.4
	1703	110.4	6.7	281.5	2028.0	7.2*	85.4
	4505	65.6	5.6	863.0	6316.3	7.3*	90.7
Down regulated	8512	59.9	4.1	1498.5	265.4	0.2	86.8
	5606	89.8	4.9	2258.6	412.8	0.2	90.5
	1407	41.6	6.8	702.6	322.7	0.5	70.6
	2701	92.6	6.4	847.8	494.9	0.6	96.9
	5502	68.2	5.2	1657.7	977.6	0.6	70.2
	4601	73.6	5.8	486.1	301.7	0.6	75.9
	9601	77.7	4.1	193.3	141.5	0.7	86.7

^aSSP - standard spot number denotes the same protein spot on different gels.^bMW - theoretical molecular weight (kDa) obtained from PDQuest software.^cpI - theoretical isoelectric charge (pH) obtained from PDQuest software.^dOD - optical density measured from the densitometer.^eCV - correlation of variation must be $\geq 70\%$; indicates how close of individual gel image match within the replicate group with the same sample and gel electrophoresis.* ≥ 2 -fold change.

Comparative 2-DE proteome expression profiles between untreated and treated CCRF-CEM. Table 6 represented quantitative analysis of proteome profiles between untreated and 5-Aza/TSA-treated HL-60 by 2-fold change.

90 differential expressed proteins were detected between untreated and 5-Aza-treated CCRF-CEM (32 upregulated and 58 downregulated protein spots). Whereas, 51 differential expressed proteins were detected between untreated and

TSA-treated CCRF-CEM (22 upregulated and 29 downregulated protein spots). Figures 5 and 6 showed representatives of 2-DE gel images of upregulated and downregulated proteins between untreated and 5-Aza-/TSA-treated

CCRF-CEM. Correlation of variation (CV) of $\geq 70\%$ identified 5 and 1 upregulated protein spots between untreated and 5-Aza/TSA-treated CCRF-CEM (Tables 7 and 8, respectively).

Table 6. Quantitative analysis of protein spots detected, spots matched, matching rate and mean coefficient of variation in untreated, 5-Aza- and TSA-treated CCRF-CEM.

Sample group	No. of protein spots detected	No. of protein spots matched	Matching rate ^b (%)	Mean Coefficient of variation (CV)
Untreated 1	257	74	26	66.39
Untreated 2	318	87	31	
Untreated 3	271	86	30	
5-Aza-treated 1	283	81	29	74.87
5-Aza-treated 2 ^a	278	278	100	
5-Aza-treated 3	251	82	29	
TSA-treated 1	410	56	28	69.01
TSA-treated 2 ^a	194	194	100	
TSA-treated 3	377	53	27	

^a5-Aza-treated 1 and TSA-treated 1 denoted as a master gel (good protein spots intensity) by PDQuest software.

^bMatching rate represents the relativeness of protein spots on a particular gel correspond spots on the master gel. Independent *t*-test, ($p < 0.05$).

Figure 5. Representative of 2-DE gel images: (A) The upregulated protein spots (i), 5-Aza-treated and (ii), untreated CCRF-CEM. (B) The downregulated protein spots (i), 5-Aza-treated and (ii) untreated CCRF-CEM. X dots denote the upregulated or downregulated protein spot label with standard spot number (SSP). The same SSP represents the same protein spot approximately on the same location on different gels. (Results from PDQuest 2-D analysis).

Figure 6. Representative of 2-DE gel images: (A) Upregulated protein spots (i), TSA-treated and (ii), untreated CCRF-CEM. (B) Downregulated protein spots (i), TSA-treated and (ii) untreated CCRF-CEM. X dots denote the upregulated or downregulated protein spot label with standard spot number (SSP). The same SSP represents the same protein on different gels. (Results from PDQuest 2-D analysis software).

Table 7. List of differential expressed proteins identified by quantitative analysis between untreated and 5-Aza-treated CCRF-CEM.

	SSP ^a	MW ^b (kDa)	pI ^c	Spot intensity (OD ^d)		Fold difference (Relative to Untreated)	CV ^e (%)
				Untreated	5-Aza- treated		
Up regulated	4602	95.4	6.1	5997.1	6461.3	1.1	75.1
	6502	77.6	5.9	1853.6	3756.2	2.0*	71.3
	6604	87.1	5.9	1359.2	3415.6	2.5*	86.5
	3101	35.7	6.1	266.6	1230.0	4.6*	94.4
	2603	86.0	6.4	792.8	4276.7	5.4*	82.4
	6305	57.7	5.9	3018.5	18592.4	6.2*	94.6
Down regulated	2201	49.3	6.25	15567.5	422.8	0.1	91.8
	8902	145.1	5.19	6487.3	1340.1	0.2	74.6
	1005	31.9	6.70	2049.8	1497.7	0.7	82.4
	7302	67.4	5.60	2524.4	1896.4	0.8	75.7
	2402	73.0	6.31	663.8	619.9	0.9	95.8

^aSSP - standard spot number denotes the same protein spot on different gels.

^bMW - theoretical molecular weight (kDa) obtained from PDQuest software.

^cpI - theoretical isoelectric charge (pH) obtained from PDQuest software.

^dOD - optical density measured from the densitometer.

^eCV - correlation of variation must be $\geq 70\%$; indicates how close of individual gel image match within the replicate group with the same sample and gel electrophoresis.

* ≥ 2 -fold change.

Table 8. List of differential expressed proteins identified by quantitative analysis between untreated and TSA-treated CCRF-CEM.

	SSP ^a	MW ^b (kDa)	pI ^c	Spot intensity (OD ^d)		Fold difference (Relative to Untreated)	CV ^e (%)
				Untreated	TSA- treated		
Up	6501	82.4	5.5	960.2	1262.4	1.3	94.8
regulated	6302	69.1	5.5	416.9	1702.8	4.1*	85.6
Down	4407	69.4	5.7	10000.4	875.6	0.1	95.1
regulated	3302	65.7	5.9	1411.9	824.6	0.6	85.2
	2204	51.6	6.1	11525.4	6952.8	0.6	80.6

^aSSP - standard spot number denotes the same protein spot on different gels.^bMW - theoretical molecular weight (kDa) obtained from PDQuest software.^cpI - theoretical isoelectric charge (pH) obtained from PDQuest software.^dOD - optical density measured from the densitometer.^eCV - correlation of variation must be $\geq 70\%$; indicates how close of individual gel image match within the replicate group with the same sample and gel electrophoresis.* ≥ 2 -fold change.

DISCUSSION

Proteomic analysis by 2-DE technique was widely applied for protein screening in cells and tissues derived from healthy and disease state (Anagnostopoulos *et al.*, 2005). HL-60 and CCRF-CEM human leukemic cell lines were extensively used as *in vitro* models in cancer research, for example to investigate anticancer drug action and gene/protein analysis (Fard *et al.*, 2012; Perri *et al.*, 2014; Wang *et al.*, 2014). Both cell lines activated proto-oncogenes that lead to cell immortalization (Drexler *et al.*, 2000; Liu *et al.*, 2014). The main objective of this study was to screen the alteration of proteome profiles of HL-60 and CCRF-CEM cell due to epigenetic treatment by 5-Aza and TSA.

This preliminary 2-DE proteomic analysis consist of total protein expression in leukemia and treatment state. Differential expressed proteins between untreated and 5-Aza/TSA-treated represented their specific cellular biological and regulatory processes including cytogenetic abnormalities and transcriptional activity (eg. upregulated, downregulated or silenced) (Heerboth *et al.*, 2014; Kornblau *et al.*, 2009). 2-DE technique has the ability to separate and analyze hundreds of protein spots based on spot counting. It might be concluded that 2-DE technique is a basis for global proteome analysis.

The protein spots separation demonstrated a distinct concentration in untreated and 5-Aza/TSA-treated HL-60 and CCRF-CEM using IPG strip pH 4-7. This result was consistent with previous study which reported that cytoplasmic protein profile of mononuclear bone marrow cells has good protein separation using IPG strip pH 4-7 (Pizzatti *et al.*, 2006). Furthermore, Coomassie blue is estimated to 8-29 ng of sensitivity staining. This staining was good for low copy number of proteins, reducing background efficiently, environmental friendly and reliable with computer software (Garfin, 2003; Lopez, 2007).

2-DE proteome profiles of untreated, 5-Aza and TSA-treated HL-60 and CCRF-CEM showed 52-79% of matching rates. The matching rate must be more than 50% to ensure that the protein spots were highly sensitive to be detected, noise was successfully reduced and maximum number of successfully matched spots were detected across all duplicate gels. Matching rate below 90% may be caused by discrepancy in term of technical inconsistencies (e.g. isoelectric focusing, gel electrophoresis, staining, alignment and spot detection) and biological factors (e.g. biochemical events, physiologic condition, timing of extra cellular signals receive and concentration of antagonist) (Bland *et al.*, 2006; Pizzatti *et al.*, 2006; Smith *et al.*, 2001).

Majority of protein spots on 2-DE gel images represented a single protein. However, some proteins expressed multiple protein spots due to

ineffective protein migration or co- and post-translational modifications (Wang *et al.*, 2003). This causes challenges in quantitative comparison of protein spots. Therefore, to overcome this problem, each individual protein spots was manually visualized to confirm the localization on the gel.

The 2-DE proteome profiles of untreated HL-60 and CCRF-CEM represented total protein activity in metabolic pathways and cellular processes of leukemia progression. Protein expression represented dynamic products encoded; if the changes were associated with disorders or external factors, it represents the phenotype of cancer (Kornblau *et al.*, 2009). Previous study reported that most protein expressions in HL-60 are involved in cell signal transduction, cell growth, apoptosis, protein and energy metabolism (Can'e *et al.*, 2010). On the other hand, most protein expressions in CCRF-CEM are involved in signal transduction, transcription factor, DNA- and RNA-binding proteins, translation, signaling, enzymes, regulatory, antigen, carriers, differentiation and receptor activity (Anagnostopoulos *et al.*, 2005).

2-DE proteome profiles of 5-Aza/TSA-treated HL-60 and CCRF-CEM may provide a functional link between the alteration of proteome profiles and epigenetic treatment. 5-Aza and TSA induced differential expressed proteins possibly due to their anti-cancer effect. It was previously shown that 5-Aza and TSA effectively inhibited and induced apoptosis in HL-60 and CCRF-CEM cell lines (Azizee *et al.*, 2018). Previous studies reported that differential expressed proteins corresponded to the quantity of transcription for cell differentiation and apoptosis *in vitro* and *in vivo* (Can'e *et al.*, 2010; Luczak *et al.*, 2012a). Furthermore, it was suggested that differential expressed proteins between leukemia and normal samples function in cell growth, cell metabolism, apoptosis and synthesis (Braoudaki *et al.*, 2013; Hu *et al.*, 2011; Pizzatti *et al.*, 2006).

In the present study, 15 upregulated proteins in 5-Aza/TSA-treated were at least 2-fold change higher than untreated HL-60 and CCRF-CEM (Tables 4, 5, 7, and 8). 2-DE detected a 2-fold change between two samples with an acceptable rate of false positives (below 7%) (Pallen *et al.*, 2014). This quantitative comparison of 2-DE

proteome profiles of untreated and 5-Aza/TSA-treated HL-60 and CCRF-CEM demonstrated distinct changes due to epigenetic treatments. Previous studies suggested that 5-Aza directly inhibit DNMT and cytosine results in cell differentiation and apoptosis, while TSA inhibits HDACs activity leading to cancer cells inhibition (Matoušová *et al.*, 2014; Yoshida *et al.*, 1990).

5-Aza and TSA re-express the silencing of certain proteins represented by upregulation of protein spots intensity. Previous study reported that HDACi treatment in colorectal cancer cells resulted in the upregulation of some pro-apoptotic genes that play important role in extrinsic and intrinsic apoptotic pathways and downregulation of anti-apoptotic genes (Zhu *et al.*, 2004). The altered proteome profiles may be linked to the response of cells to epigenetic modification drugs. This preliminary study indicates a basis for leukemia prognostic and should have been accompanied by protein database analysis using high-throughput protein identification (e.g. mass spectrometry) to demonstrate structural and conformational characterization of the proteins of interest.

CONCLUSION

A proteomic method based on 2-DE technique was developed for qualitative and quantitative analysis of HL-60 and CCRF-CEM. 5-Aza and TSA have great potential as anti-cancer effect in HL-60 and CCRF-CEM. Comparative analyses revealed that both epigenetic modification drugs induced different expressed proteins. The differentially expressed proteins discovered in this study may provide an overview to the protein alteration in a variety of cell functions and biological processes which could provide potential information in understanding the progression of leukemia. This preliminary finding is an early stage to provide fundamental information on disease mechanism and potential treatment.

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