LAB REPORT

Introduction

Analysis of gene expression is one of the most important means by which the mRNA and/or protein profile of a cell can be analyzed. This is especially important to understand protein expression and function in different cell types, and especially in cancer cells. In several types of cancers, specific genes are either upregulated or downregulated, and this misregulation contributes to the proliferative qualities of cancer cells. Hence, by analyzing the expression of specific genes in cancer cells or tissues, the gene misregulation can be narrowed down and targeted therapies can be developed (Tarek et al. 2016).

This report analyzes the methylation status and expression of two genes, *TAL1* and *CDH1*, in two cell lines, T-cell acute lymphoblastic leukemia (T-ALL) and lung epithelial cells (LEPI) respectively. Both the *TAL1* and *CDH1* genes play important roles in tumour progression and invasion and understanding their expression profile can give several insights into the stage of the tumour.

The objective of these experiments was to analyze the methylation status and gene expression of the genes, *TAL1* and *CDH1* using the total gDNA and total RNA of the T-ALL and LEPI cell lines respectively. The MSRE assay was performed to detect cleavage by a methylation-sensitive restriction enzyme. The total RNA from these samples was used to analyze gene expression by QPCR.

It was seen that the gene *TAL1* was not expressed in either of the two samples of T-ALL which was revealed by non-cleavage of the probed sites and absence of the gene product in QPCR. In case of the LEPI samples, LEPI1 showed presence of methylation whereas LEPI2 showed methylation of one site and non-methylation of the other site. QPCR analysis of these samples revealed that the gene *CDH1* was expressed in LEPI1 and not in LEPI2.

In conclusion, the gene *TAL-1* is not expressed in the T-ALL cell line whereas the gene *CDH1* is partially expressed in the LEPI cell line.

Materials and Methods

MSRE Assay

Genomic DNA (gDNA) from the T-cell Acute Lymphoblastic Leukemia (T-ALL) clonal cell line was digested with HpaII and MspI separately. For each reaction, 100 ng of gDNA was digested with 5 units of the restriction enzyme. A control 'no enzyme' reaction was also set up where 1 µl of water was added to 100 ng gDNA instead of the enzyme. The restriction digestion reactions were incubated at 37 ˚C for 1 hour, after which they were heat inactivated at 95 ˚C for 15 minutes. The digested and undigested gDNA samples were diluted to 5 ng/µl, to be used for subsequent PCR reactions.

Gene Expression Assay

For setting up the PCR reactions, 2 µl of the gDNA samples were added to 8-well strip tubes. The 2 x SensiFAST™ SYBR® kit was used to prepare the QPCR reaction mix, and two reactions were set up for each gDNA sample, one for each of the MSRE primer pairs, T-ALL_MSRE4 and T-ALL_MSRE5. Once the reactions were set up, the samples were subjected to QPCR using the ABI 7500 FAST instrument. The QPCR reaction conditions are given in the Appendix.

Total RNA obtained from the above QPCR reaction was subjected to reverse transcription by setting up cDNA synthesis reactions using the SensiFAST™ cDNA synthesis kit. The reverse transcriptase enzyme (1 µl) was added to 5 ng of the RNA sample in the reaction mix and incubated. The synthesized cDNA was used for gene expression analysis of the genes *TAL1* and *CDH1*, and the normalization gene, *NFYB*. QPCR reactions were set up using 0.2 µM of each gene-specific primer, 1X SensiFAST™ SYBR® reaction mix, and 2 µl of cDNA in the ABI 7500 FAST instrument. The PCR cycling conditions for MSRE QPCR are given in the Appendix.

Nanopore Library Construction

The gDNA sample (1.5 ng) was subjected to a nanopore DNA amplification reaction using 0.5 µM of the reaction primers, T-ALL_1 and T-ALL_2. The PCR reaction conditions for nanopore sequencing are given in the Appendix. After completion of PCR, the PCR amplicons were subjected to DNA end repair using the end repair enzyme mix and incubating it at 20 ˚C for 10 minutes. The repaired blunt-end DNA sample was purified using a spin column and subjected to rapid ligation of sequencing adaptors. The PCR amplicon (100 ng) was ligated with 0.75 pmoles of sequencing adaptor and 1 unit of the enzyme T4 DNA ligase.

The ligated PCR amplicons were subjected to another round of PCR using an adaptor PCR primer (0.5 µM) and 1X of MyTaq reaction mix. Control reactions were set up with unligated PCR amplicons and without template DNA. The PCR reactions conditions are given in the Appendix.

Results

MSRE Assay for T-ALL and LEPI cell lines

Total gDNA from the T-ALL cell line was digested with the methylation-sensitive restriction enzyme HpaII and the isoschizomer MspI. A ‘no enzyme’ control was also set up to compare the degree of cleavage with the two restriction enzymes. The graphs of the cycle threshold values obtained after QPCR for each PCR cycle for T-ALL_1 and T-ALL_2 samples using both MSRE4 and MSRE5 primers are given below.

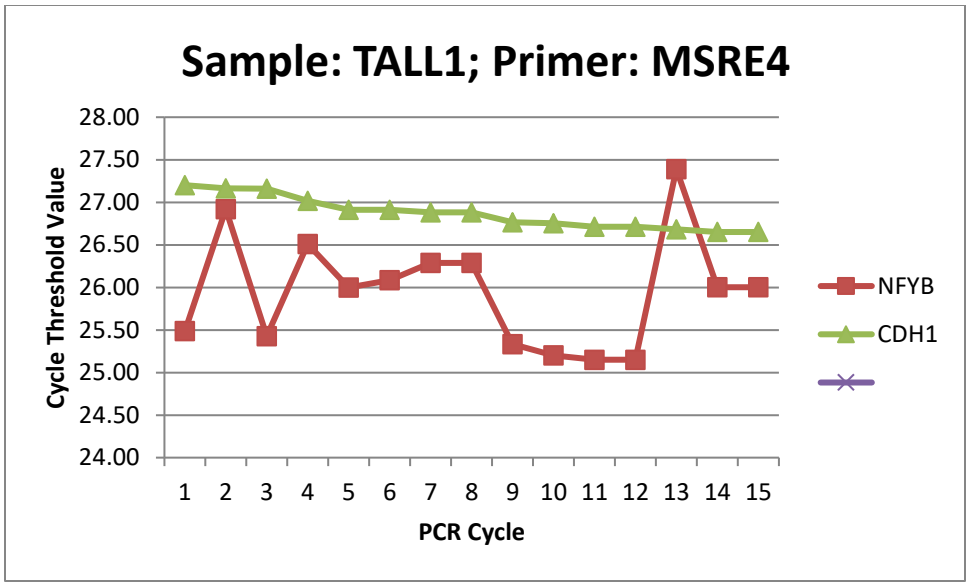

Figure 1: Cycle threshold values for T-ALL_1 using MSRE4 primer

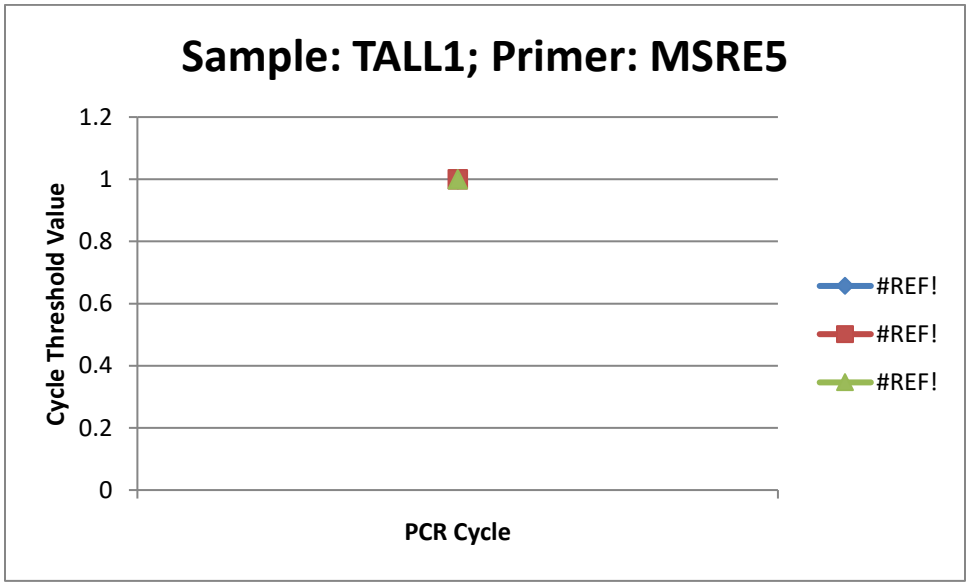

Figure 2: Cycle threshold values for T-ALL_1 using MSRE5 primer

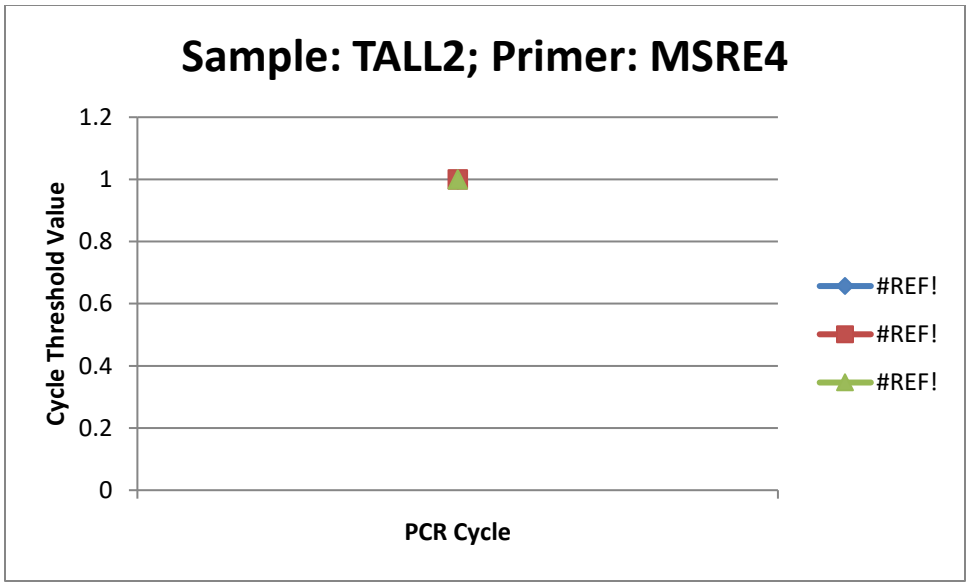

Figure 3: Cycle threshold values for T-ALL_2 using MSRE4 primer

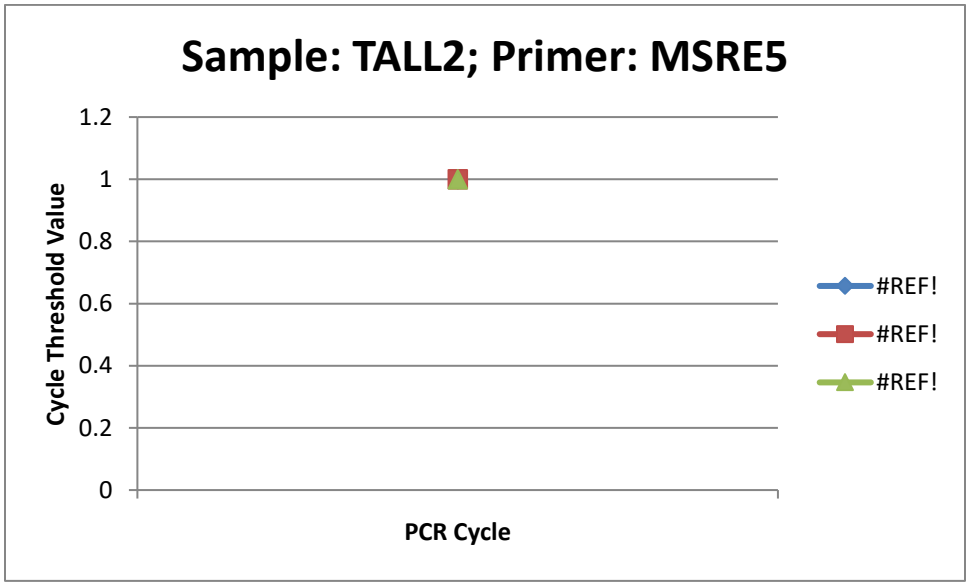

Figure 4: Cycle threshold values for T-ALL_2 using MSRE5 primer

From the above graphs, it can be seen that the T-ALL_1 gDNA is methylated at both sites MSRE4 and MSRE5 resulting in an absence of cleavage by the methylation-sensitive enzyme HpaII. In case of the T-ALL_2 gDNA sample, the MSRE4 site is methylated giving low cycle threshold values whereas the MSRE5 site is unmethylated giving high cycle threshold values.

Similarly, the MSRE assay was performed for the LEPI cell line and analyzed using the primers MSRE3 and MSRE4. The cycle threshold values for the samples LEPI1 and LEPI2 using the specific primers are given in Figures 5 to 8 below.

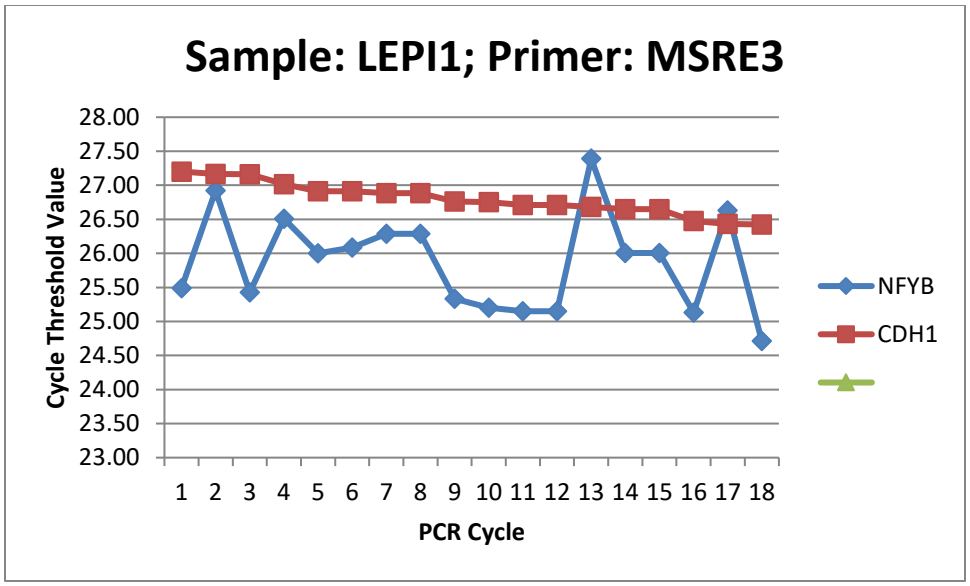

Figure 5: Cycle threshold values for LEPI Sample 1 using the primer MSRE3

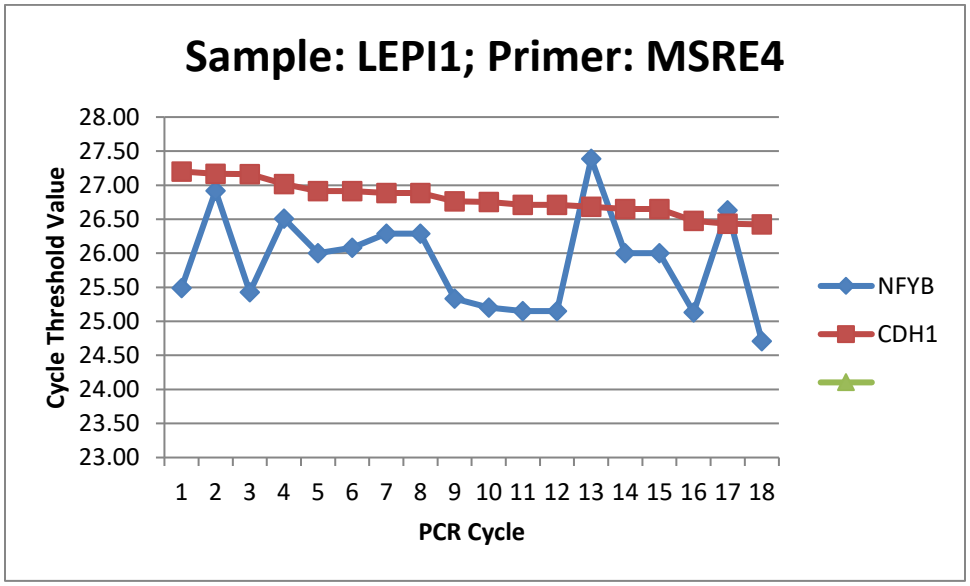

Figure 6: Cycle threshold values for LEPI Sample 1 using the primer MSRE4

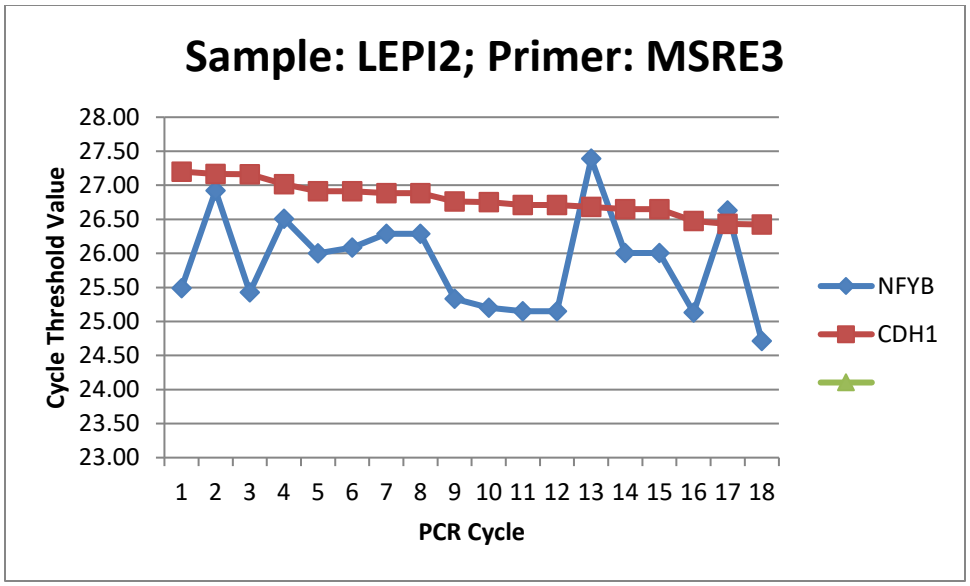

Figure 7: Cycle threshold values for LEPI Sample 2 using the primer MSRE3

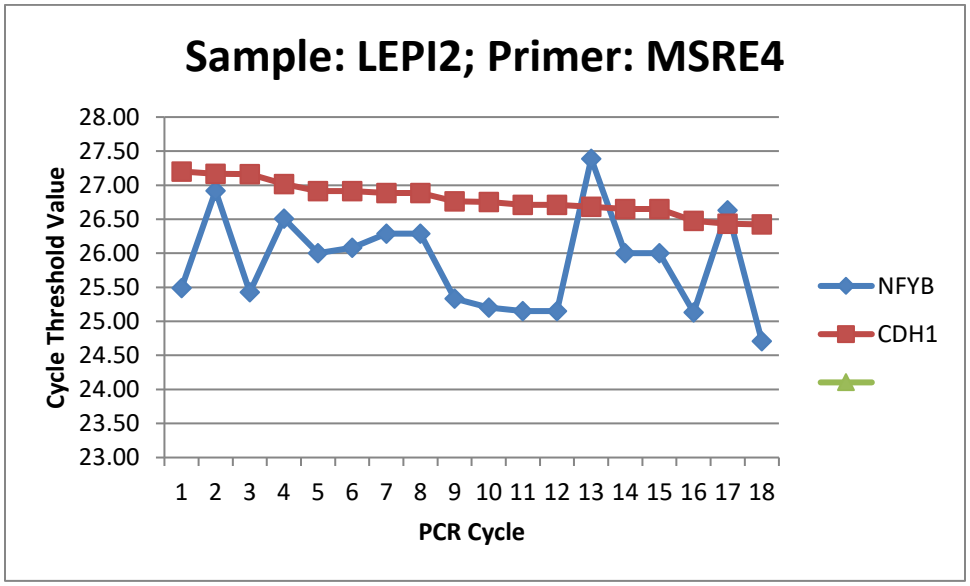

Figure 8: Cycle threshold values for LEPI Sample 2 using the primer MSRE4

From the above graphs, it can be seen that the LEPI1 gDNA sample is methylated at both sites MSRE3 and MSRE4 resulting in an absence of cleavage by the methylation-sensitive enzyme HpaII. In case of the LEPI2 gDNA sample, the MSRE3 site is methylated giving low cycle threshold values whereas the MSRE4 site is unmethylated giving high cycle threshold values.

QPCR analysis of gene expression

Total RNA from the T-ALL and LEPI clonal cell lines was used to analyze gene expression of *TAL1* and *CDH1* genes respectively. The cycle threshold values for T-ALL_1 and T-ALL_2 samples for the amplification of *TAL1* and the normalization gene *NFYB* are given in figures 9 and 10 below.

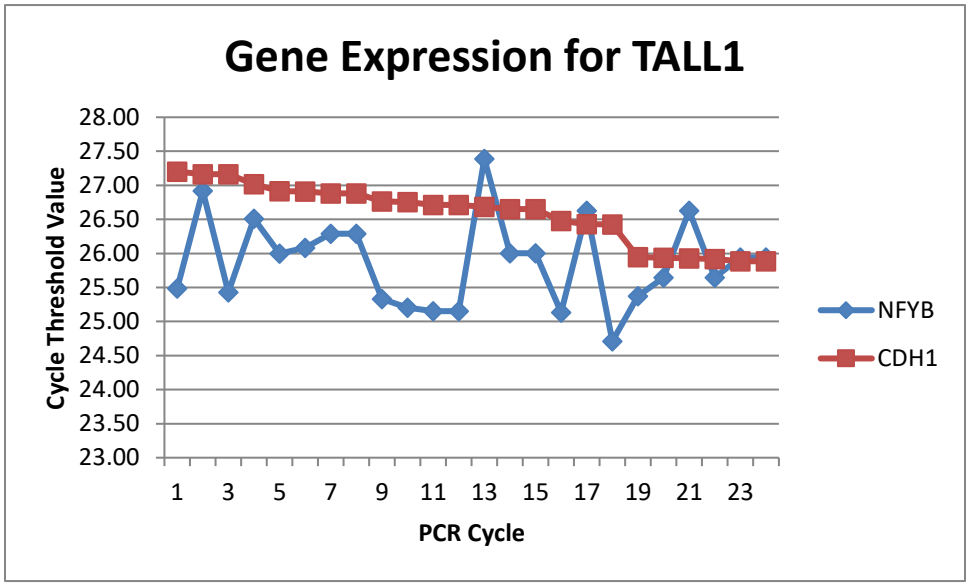

Figure 9: Cycle threshold values for T-ALL_1 sample for gene expression analysis of *TAL1*

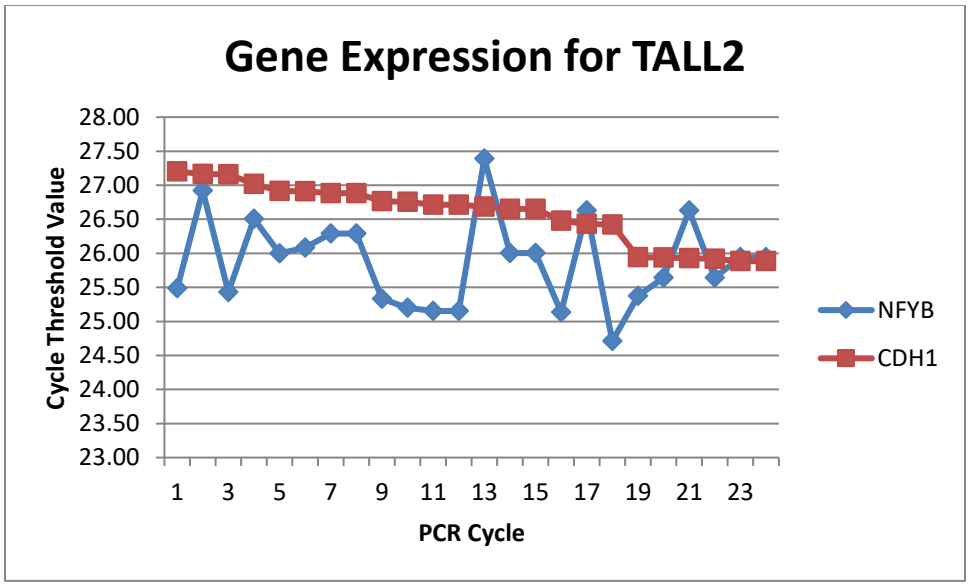

Figure 10: Cycle threshold values for T-ALL_2 sample for gene expression analysis of *TAL1*

Similarly, the gene expression analysis of *CDH1* gene in LEPI1 and LEPI2 samples was performed and the cycle threshold values are given in Figures 11 and 12 below.

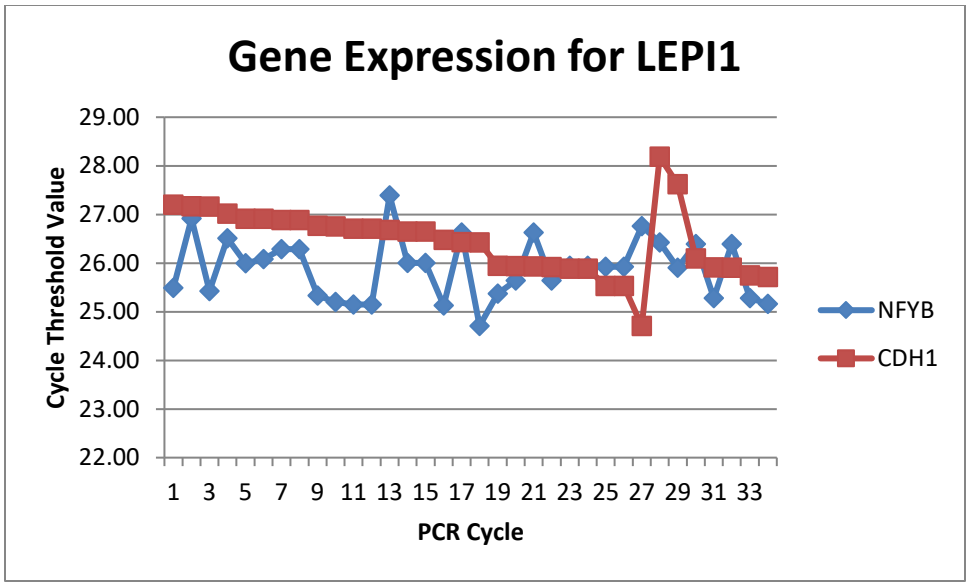

Figure 11: Cycle threshold values for LEPI1 sample for gene expression analysis of *CDH1*

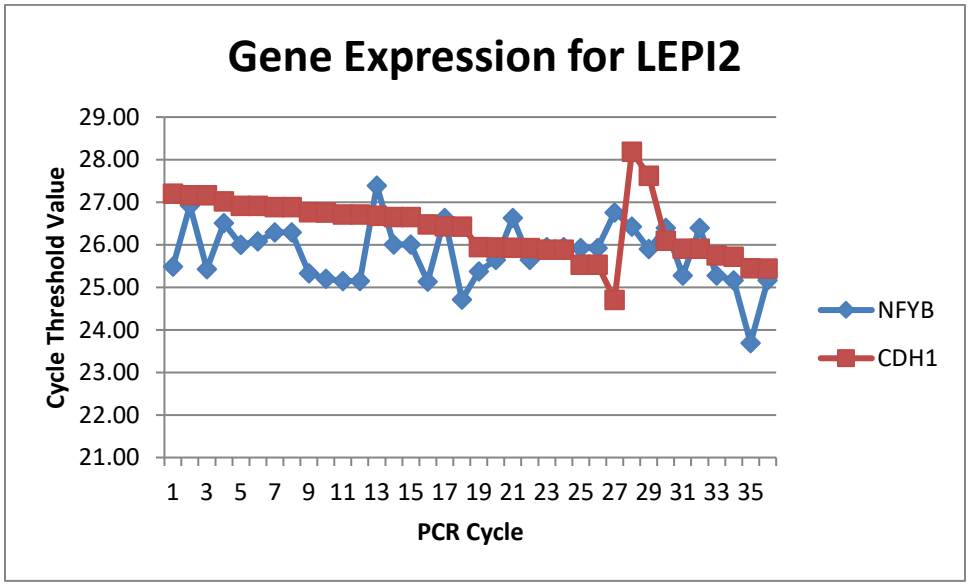

Figure 12: Cycle threshold values for LEPI2 sample for gene expression analysis of *CDH1*

From the figures 9, 10, 11, and 12 above, it is evident that the gene *TAL1* is not expressed in both the T-ALL_1 and T-ALL_2 samples based on the low cycle threshold values for both the samples in comparison to the normalization gene *NFYB*. In the case of LEPI RNA samples, the gene *CDH1* shows high cycle threshold values in sample 1 and low cycle threshold values in sample 2 indicating that the *CDH1* gene is expressed in LEPI1 and not in LEPI2.

Discussion

One of the most important regulatory mechanisms for gene expression adopted by cells is the addition of methyl groups to cytosine bases at certain specific locations. This is often considered the fifth nucleotide in the genome and its presence in genes gives several insights into cell-specific gene expression patterns (Varley et al. 2013).

TAL1 is a transcription factor that regulates hematopoiesis in the body and this gene is often found to be misexpressed in T-cells in the case of T-cell acute lymphoblastic leukemia (T-ALL). Hence, analysis of the *TAL1* gene expression is important in this cancer as it can give several insights into the misregulation of T-cell development leading to T-ALL (Tan et al. 2019). In this report, it has been proved that the gene *TAL1* is not expressed in two samples of the T-ALL clonal cell line when probed using the MSRE assay and QPCR. This is in contrast to published literature and different studies have shown that an overexpression of the gene *TAL1* is a hallmark of T-ALL (Correia et al. 2016).

The *CDH1* gene codes for an epithelial-cadherin protein that is involved in calcium-dependent cell-cell adhesion in lung epithelial cells. Due to its several functional roles in the body, it has been shown that its downregulation or suppression plays an important role in tumour invasion. A reduced expression of *CDH1* gene and hypermethylation of its promoter has been reported in many tumours such as breast, gastric, colorectal, melanoma, squamous cell carcinoma, and hepatocellular carcinoma (Gall & Frampton 2013).

In this report, the two samples used for analysis have given contrasting results. In case of methylation analysis, it is seen that LEPI1 is methylated at both sites indicating a downregulation of the gene, whereas only one of the sites is methylated in LEPI2 indicating that some expression of the gene has occurred. Comparing this with the results of the QPCR analysis, it is seen that the *CDH1* gene is expressed in LEPI1 and not expressed in LEPI2. The variations in the results can be attributed to human errors while handling the samples, enzymes, and primers.

References

Correia, N C, Melao, A, Povoa, V, Sarmento, L, de Cedron, M G, Malumbres, M et al. 2016, 'microRNAs regulate TAL1 expression in T-cell acute lymphoblastic leukemia', *Oncotarget*, vol. 7, no. 7, pp. 8268–8281.

Gall, T M H & Frampton, A E 2013, 'Gene of the month: E-cadherin (CDH1)', *J Clin Patho*, DOI 10.1136/jclinpath2013-201768.

Tan, T K, Zhang, C & Sanda, T 2018, 'Oncogenic transcriptional program driven by TAL1 in T-cell acute lymphoblastic leukemia', *International Journal of Hematology*, vol. 109, no. 1, pp. 5–17, DOI 10.1007/s12185-018-2518-z.

Tarek, S, Elwahab, R A & Shoman, M 2017, 'Gene expression based cancer classification', *Egyptian Informatics Journal*, vol. 18, no. 3, pp. 151–159, DOI 10.1016/j.eij.2016.12.001.

Varley, K E, Gertz, J, Bowling, K M, Parker, S L, Reddy, T E, Pauli-Behn, F et al. 2013, 'Dynamic DNA methylation across diverse human cell lines and tissues', *Genome Research*, vol. 23, pp. 555–567, DOI 10.1101/gr.147942.112.

Appendix

MSRE QPCR Cycling Conditions

Cycle Number	Temp (˚C)	Time	Comments
1	95	5 min	Polymerase Activation
2 - 40	95	10 sec	Denaturation
	62	10 sec	Annealing
	72	30 sec	Extension and collection of fluorescence data

PCR Cycling Conditions for Nanopore Sequencing

Step	Temperature (˚C)	Time
Initial Denaturation	95	1 min
10 Cycles	95	15 sec
	Begin 10 ˚C above primer annealing temperature and then decrease 1 ˚C per cycle	15 sec
	72	2 mins
25 Cycles	95	10 sec
	Primer annealing temperature	10 sec
	72	2 mins
Final Extension	72	2 mins
Hold	10	-

Sequencing Adaptor Primer Sequence

5’ CGACGTCGACTATCCATGAACAGCGC 3’

3’ GTACTTGTCGCG 5’