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Epigenetic Alterations of *DLL4* and *Hes5* in Acute Lymphoblastic Leukemia (ALL)

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Abstract

Acute lymphoblastic leukemia (ALL) is a hematologic condition with more than a quarter of pediatric cancers. Aberrant promoter methylation of Notch pathway genes causes the deactivation of TSGs. The pathway is also considered a crucial factor in the pathogenesis of ALL due to its active involvement in B and T cell development. Hypermethylation of Notch pathway genes has been reported previously. In this study, the promoter methylation frequency of genes DLL4 and Hes5 of the Notch pathway were studied using methylation specific PCR in 30 pediatric ALL blood samples against 10 healthy controls. The objective of the study was to find the subtype specific diagnostic biomarker for ALL. Hypermethylation frequency of DLL4 in pre-B ALL and T-ALL samples was found to be 84.21% and 100%, respectively. Whereas, Hes5 showed 100% mixed methylation in both diseased and control samples. The results predicted the possible epigenetic changes of Notch pathway and the possible role of DLL4 as a diagnostic biomarker of ALL.

Keywords: acute lymphoblastic leukemia (ALL), *DLL4, Hes5,* epigenetic, *Hes5*, *DLL4,* epigenetic, methylation specific PCR promoter methylation, tumor suppressor genes

Introduction

Leukemia is 15th most commonly diagnosed cancer and 11th predominantly leading cause of cancer mortality worldwide [1]. Acute lymphoblastic leukemia represents more than a quarter of all pediatrics cancers, causes proliferation of immature lymphoid cells and it the basic characteristic of this malignancy, which takes place in bone marrow, peripheral blood, and other organs. Historically, based on clinical data and morphology of the malignant cells, leukemia was classified into four groups one of them is acute lymphoblastic leukemia [2].

In acute leukemia, ALL is the most prevalent malignancy with a frequency of 75%–80% in children. In the United States in 2019, the American Cancer Society reported 5,930 new cancer cases and 1,500 deaths. SEER cancer statistics review

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from 1975-2015 reports the incidence rate of ALL as 1.38 per 100,000 individuals per year [3, 4]. In Pakistan according to Shaukat Khanum Memorial Cancer Hospital reports ALL was most frequently occurring malignancy in children with 1975 cases (20.2%) from Dec 1994 to Dec 2019, and according to the 2020 GLOBOCAN report 474,519 new cases and 311,594 deaths were reported due to leukemia.

Cancer is an abnormality that is controlled by series of genetic alterations, gross numerical and structural chromosomal abnormalities that regulate development and cell cycle [5, 6]. In the diagnosis of ALL, CBC and BM biopsy are preliminary tests and detection of at least 25% blasts indicate that the cells are leukemic which are just 5% in normal condition but still just morphological testing does not provide a specific diagnosis. High-profile tests such as cytogenetic FISH, immunophenotyping, flow cytometry, RT-PCR, SNP analysis and genomic hybridization are needed for confirmation and early diagnosis of disease. As cancer is characterized by genetic and epigenetic abnormalities, and in epigenetics more specifically hypermethylation have been reported very extensively in ALL and many other cancers pathogenesis [7].

The aim of this study is to find subtype specific diagnostic biomarkers and to replace protein-based biomarkers with cost effective diagnostic markers. As in protein expression studies expensive antibodies are required for expression studies and protein expression studies are also more time consuming. So, successful validation of some effective disease specific biomarkers can be a hotspot in ALL diagnosis and therapeutics.

Constitutive Notch signaling results in transformation of progenitor cells of T-lymphocytes into neo-plastic cells and leads to malignancy. Over-expression of Notch3 is reported in nearly all human acute lymphoblastic T cell subtype [8, 9]. *DLL4* being an important regulator of development, growth and being a vital influencer of Notch signaling and T cell development at apoptotic and proliferative level also regulates T-cell lymphoproliferative disease by its over-expression [10]. Similarly, epigenetic regulation of *Hes5* gene has also been associated with ALL. Activated *Hes5* causes cell differentiation in multiple tissues, whereas abnormal regulation and expression of *Hes5* can cause developmental abnormalities.

For carrying the present study, Notch pathway genes *Hes5* and *DLL4*were selected to determine their role in the pathogenesis of ALL. Promoter methylation frequency of genes *DLL4* and *Hes5* of the Notch pathway were studied by

methylation specific PCR (qMSP) in 30 pediatric ALL blood samples against 10 healthy controls.

Methodology

Sample Collection

Fresh blood sampling from pediatric ALL patients was carried out form "Children's Hospital, Lahore" from November 2018-April 2019. Samples were collected in EDTA vials and immediately stored at -4ºC. Study was approved by Ethical committee Board of Children's Hospital. Prior to sample collection, written consent was obtained from patient's guardian and clinical data of patients was obtained from cancer registry of hospital. In the present study, most prevalent subtype was pre-B subtype with a few pre-T subtype ratios and the age of patients included in the study was in the range of between 1.5-15 years.

Primer Designing

Primers of the selected genes (*DLL4, Hes5*) were designed in accordance to their gene promoter region. Promoter sequences of both genes were obtained from (EPD) eukaryotic promoter database while accession number of *DLL4* and *Hes5*are NM019074 and NM001010926 respectively. UrogeneMethPrimer software version 2.0 was used for designing primers which generated two primer pairs (methylation specific and unmethylation specific primers) after insilico conversion of promoter sequence of both genes. Primer sequence and thermodynamic properties of *DLL4* and *Hes5* are given in table 1 and 2 respectively.

DNA Extraction and Bisulfite Modification

Organic-phenol DNA extraction (manual) method was used for carrying out DNA extraction from all samples (diseases + healthy controls). For checking the DNA concentration of extracted samples spectrophotometer (Nanodrop™) was used which indicated DNA concentration range between 100-1800ng/µl. Qiagen-Epitect Fast DNA Bisulfite kit™ was utilized for sodium bisulfite conversion of all samples.

Quantitative Methylation Specific PCR

Quantitative methylation specific PCR was carried out for analyzing methylation patterns from *DLL4* and *Hes5* gene promoter regions. Real-time CFX96 detection system was utilized for qMSP. After optimization, qMSP profile for both genes was set according to standard conditions as: initial denaturation for 10 min at 95^0C followed by 40 cycles of denaturation for 30sat 95^0C. Primer annealing and extension was done at 55^0C for 30s. Melt curve analysis was also carried out at

Table 1. Thermodynamic Properties of *DLL4* Primers

Gene	Primer ID	Primer Sequences	Primer Length	Tm (◦C)	GC %	Size of Product
DLL4	MF	5’ GTTATTACGGAGGATTGGTTATTTC3’	25	58.41	64.00	167
	MR	5’ ACCTCTAACTACTACAATCCCAACG3’	25	59.11	64.00	
	UF	5’TTATTATGGAGGATTGGTTATTTTG3’	25	57.64	64.00	166
	UR	5’ ACCTCTAACTACTACAATCCCAACAC3’	26	58.26	65.38	

Table 2. Thermodynamic Properties of *Hes5* Primers

Gene	Primer ID	Primer Sequences	Primer Length	Tm (◦C)	GC %	Product Size
Hes5	MF	5’GTTTATTTGTTTTTTGGGGAGC 3’	22	58.95	72.73	154
	MR	5’GACCTAACGCCTAAAATCCG 3’	20	58.72	80.00	
	UF	5’GTTTATTTGTTTTTTGGGGAGTGT 3’	24	59.60	75.00	154
	UR	5’AACCTAACACCTAAAATCCA3’	20	51.50	80.00	

MF (methylation specific forward), MR (methylation specific reverse), UF (unmethylation specific forward), UR (unmethylation specific reverse)

65^{0}C-95^{0}C for 0.05s with increment of every 0.5^{0}C. Thermal Cycler™ was used for amplification while Bio-Rad CFX manager software was utilized for analyzing final results followed by agarose gel electrophoresis (2%) for further confirming and resolving the amplified products. Syngene™ documentation system was utilized for capturing gel images. Recipe of methylation specific PCR with their used and final volume is given is table 3.

Table 3. Recipe of Methylation Specific Real-Time PCR Reaction

Components	Volume used (10 µl)	Final concentration
DNA template	1.5 µl	200ng
Forward primer	0.3 µl	0.2 µM
Reverse primer	0.3 µl	0.2 µM
SYBER green (master-mix)	5 µl	1X
Nuclease-free water	2.5 µl	NA

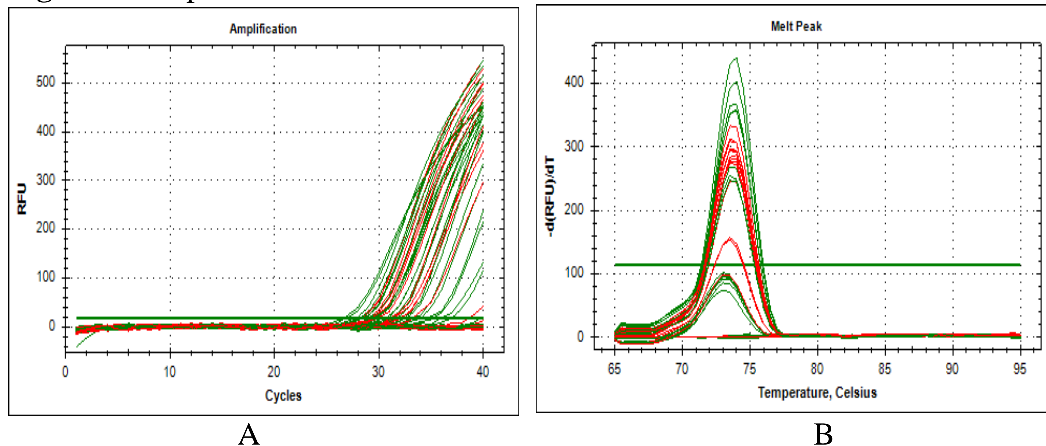
Results

Manual DNA extraction from patients as well as healthy controls was done and Qiagen methylation kit™ was used for bisulfite modification of all extracted samples. Quantitative real-time methylation specific PCR was performed for analyzing promoter methylation of both genes (*DLL4* and *Hes5*) from 30 ALL samples (19 B-ALL samples, 11 T-ALL) against 10 healthy controls. For promoter methylation evaluation, positive and negative controls were also run whereas non-template controls (NTC) were run for contamination assessment. For confirmation of results, final amplified products were run on 2% agarose gel.

qMSP for *DLL4*

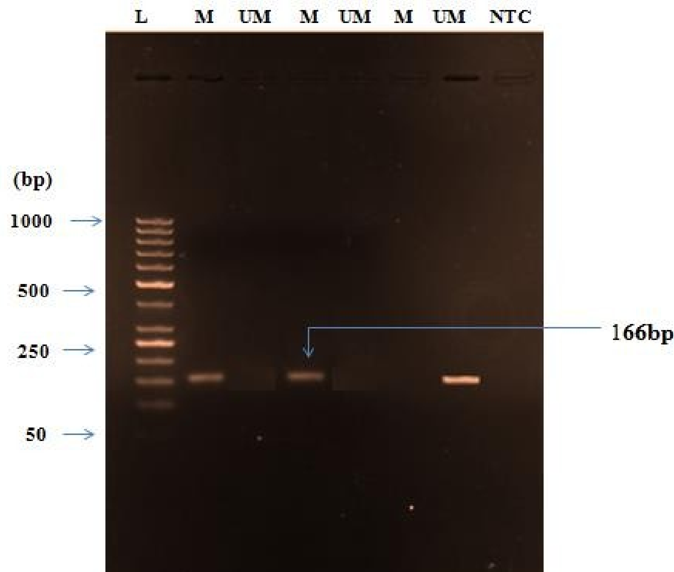
Results of qPCR indicated that out of 19 B-ALL samples 84.21% were methylated and 5.2% were un-methylated whereas T-ALL samples showed 100% methylation. Healthy controls indicated 100% unmethylation of samples. Results of hypermethylation in ALL samples and hypomethylation in healthy samples are given in the table 4. Melt curve analysis indicates the specificity of the amplified PCR products of *DLL4* as only one single peak of melting curve was indicated by CFX manager at 73.5^{0}C. Melt curve peaks and amplification curves are given in image 1 whereas 2% agarose gel results of final products are given in image 2.

Figure 1. Amplification Curves and Melt Curves of M-*DLL4* and UM-*DLL4*



(A) Collective amplification curve of M-*DLL4* and UM-*DLL4*. (B) Collective melt curves of M-*DLL4* and UM-*DLL4* are representing the specificity of the product. Green color: methylation, Red color: Unmethylation. M: Methylated, UM: Un-methylated.

Figure 2. Amplified PCR Products of M-*DLL4* and UM-*DLL4* Resolved on 2% Gel



Sample 1 was loaded in well 2 and 3, sample 2 was loaded in well 4 and 5, sample 3 was loaded in well 6 and 7 and well 8 was loaded with non-template control, L: Ladder M: Methylated, UM: Unmethylated. NTC: non-template control

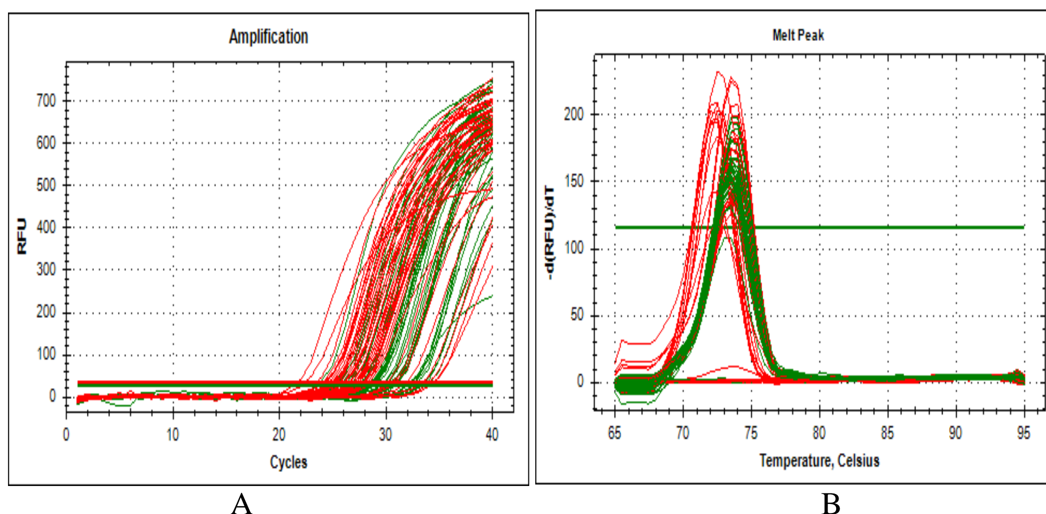
Table 4. Methylation Status of *DLL4* qPCR Results Details

Sample Type		**Methylation**	**Unmethylation**
Patient samples (No. 30)	B-ALL (No.19)	84.21%	5.2%
	T-ALL (No.11)	100%	0%
Healthy controls (No. 10)		0	100%

qMSP for *Hes5*

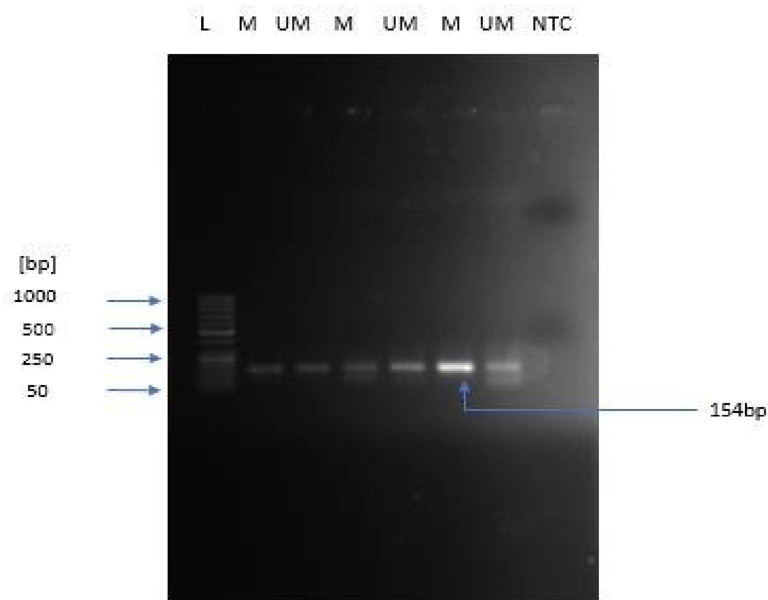
qMSP results for *Hes5* indicated 100% mixed methylation. Specificity of the products was analyzed by melt curve analysis of the samples and melting peaks of methylated-*Hes5* and unmethylated-*Hes5* appeared between 72^{0}C and 74^{0}C. Amplification curves and melt curve peaks of *Hes5* are given in figure 3 and agarose gel image of *Hes5* amplified products is given in figure 4.

Figure 3. Amplification Curves and Melt Curves of M-*Hes5* and UM-*Hes5*



(A) Collective amplification curve of methylated *Hes5* and unmethylated *Hes5*. (B) Collective melt peaks of methylated *Hes5* and unmethylated *Hes5* and are representing the specificity of the products. Green: methylation, red: Unmethylation.

Figure 4. Amplified PCR Products of M-*Hes5* and UM-*Hes5* Resolved on 2% Gel



Sample 1 was loaded in well 2 and 3, sample 2 was loaded in well 4 and 5, sample 3 was loaded in well 6 and 7 and well 8 was loaded with non-template control L: Ladder M: Methylated, UM: Unmethylated. NTC: non-template control.

Discussion

Leukemia is a disease characterized by both genetic and epigenetic factors while in epigenetic modifications, hypermethylation have been extensively involved in suppression of TSGs. Symptoms of this disease in general are various and nonspecific. In children, prolonged fever, bone pain (23%), infections, petechiae, easy bruising, and disruption of normal hematopoiesis, whereas fever, night sweats, weight loss are its fundamental symptoms [11, 12].

Epigenome of humans basically consist of three main components. Histone modifications, changes in DNA methylation level and RNA interference are interlinked to each other at epigenetic level. Changes at epigenetic level are reversible and most common methylation alternations like promoter hypermethylation has been reported in human cancer through disruption of TSGs [13, 14].

In this regard study of epigenetic alterations that are effectively involved in the pathophysiology of cancers has been eagerly studied in previous 10 years. Methylation across CGIs in all genetic subtypes of ALL has been linked to the prognosis and disease progression [15]. Epigenetic studies can pave a path for valuable study of disease pathways and also help in predicting and formulating the potential therapeutic targets for diverse subtypes of ALL [15].

Biological output of Notch pathway is interlinked to events that happens during cellular communication and its activation is highly scrutinized through various steps. Notch signaling plays dual role in ALL and is abrogated due to some unknown reasons. Hypermethylation of these genes can be the possible cause of abrogation as this hypothesis is supported by *Quang* and his colleagues in their study [16]. Notch pathway regulates cell to cell communication, self-renewal of stem cells of blood and influences multiple steps such as cell differentiation and maturation [17-19].

Constitutive Notch signaling results in transformation of progenitor cells of T lymphocytes into neoplastic cells and leads to malignancy but in a variety B-cell leukemic tissues Notch signaling is reported as a tumor suppressor. Growth arrest and apoptosis are induced by continuous expression of Notch receptors (ICN1-4) or their downstream target [20]. *Hes5* (a downstream target of Notch) inhibits the promoter gene expression of *FBXW7β* and results in prolonged signaling by creating a positive feedback. Activated *Hes5* causes cell differentiation in multiple tissues whereas abnormal regulation and expression of *Hes5* can cause developmental abnormalities [21]. Consistent expression of *DLL4 (*delta like-4) is found in lymphocytic and blood producing organs but T cell development is abrogated when *DLL4* is not expressed on thymic epithelial cells (TEC). Thus the dual behavior of Notch signaling is possibly controlled by epigenetic regulation of genes of the pathway, still the exact molecular basis behind such behaviors is not clear and needs more in depth study [16].

In this study, methylation level of genes of the Notch pathway *Hes5* and *DLL4* in patient samples (n=300 against controls (n=10) by methylation-specific PCR was studied. Methylation studies revealed hypermethylation of *DLL4* in 84.2% in B-ALL blood samples whereas only one sample showed hypomethylation in this subtype and in normal controls there was 100% hypomethylation. On the other hand, T-ALL samples also showed 100% hypermethylation suggesting *DLL4* as an effective biomarker for diagnosis of ALL. In contrast to previous studies, *Hes5* was found preferentially hypermethylated in both patient samples and healthy controls showing mixed

methylation while in previous studies *Hes5* was found to be hypermethylated only in B-cell specific cell lines and very low methylation of T-cell specific cell lines were observed.

Unlike previous study our results are different and these may be due to different study population. This study is further strengthening the concept that Notch pathway genes may function as TSGs in both subtypes and epigenetic regulation of Notch pathway genes must be studied in both lineages instead of just restricting these biomarkers to just one subtype. It also helps in the targeted therapy of ALL by regulation of tumor-suppressive role of this \ pathway in both subtypes. As previously, the main focus was on targeting the point mutations in desregulation of Notch signaling (T-ALL) reported by Pelullo et al. but epigenetic factors can also be the possible contributor toward deregulation so *DLL4* may act as a significant factor in T-ALL therapeutics [22]. So effective studies on patient, control and post treatment samples are required to further validate the role of these biomarkers in ALL and their specific contribution to a specific subtype either B-ALL or T-ALL.

Conclusion

Epigenetic markers provide the benefit of early diagnosis as genetic and transcriptomic event occurs at later stages than does epigenetic events so it's a dire need to study these markers for early diagnosis and better prognosis of disease. In our present study, the observation of hypermethylation of these two genes of the Notch pathway indicated important role of the epigenetic alterations of the pathway in ALL progression. The methylation behavior of *DLL4* and *Hes5* in both healthy controls and patients can be studied further for its anomalous behavior while *DLL4* can be reported as a significant biomarker as this much higher level of methylation of *DLL4* has not been previously reported in the literature.

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Supplementary Table on inclusion criteria of samples.

Patient No.	Age (Y)	Sex	Diagnosis	CBC				DLT			Blast Cells %	CNS Involvement
				RBC $\times 10^6$/ul	Hg g/dl	WBC $\times 10^3$/ul	PLT $\times 10^3$/ul	L %	N %	M %		
•	13	M	Pre-B	3.21	9.0	46.2	90	20	8	2	-	No
•	9	M	Pre-B	1.27	3.2	20.9	14	70	25	7	30	No
•	5	M	Pre-B	3.24	10.7	3.6	48	38	-	13	40	No
•	4	M	Pre-B	4.47	13.2	0.8	14	-	-	-	-	No
•	1.5	F	Pre-B	2.21	12	21.5	371	96	3.2	-	-	No
•	5	F	Pre-B	2.34	7.2	84	37	35	8	5	50	No
•	7	M	Pre-B	2.70	8.6	11.2	44	20	10	2	68	Yes
•	2.5	M	Pre-B	2.81	5.6	73.5	32	94	-	-	89	No
•	3.6	M	Pre-B	3.30	10.5	1.5	30	76	14	-	-	No
•	12	F	Pre-B	2.29	7.3	12.2	91	25	12	3	60	No
•	10	F	Pre-B	2.48	8.6	2.3	199	62	30	4	60	No
•	9	M	Pre-B	2.55	6.0	58.3	155	78	12	2	-	No
•	10	M	Pre-B	4.36	10.2	11.2	46	65	5	8	20	No
•	3	F	Pre-B	5.80	14.1	32.8	419	28	6	-	80	No
•	14	M	Pre-B	3.6	10.9	173.6	153	6	20	4	70	No
•	1.4	M	Pre-B	1.63	4.5	153	10	76	-	-	-	No
•	2	F	Pre-B	5.54	17.2	9.3	16	40	15	3	40	No
•	11	M	Pre-B	1.53	5.0	227	83	78	-	-	90	No
•	12	M	Pre-B	3.46	10.3	45	85	45	-	3.8	25	No
•	12	F	Pre-T	2.78	8.2	84	20	78	-	2	75	No
•	3	M	Pre-T	3.36	8.1	137.2	82	63	-	17	80	No
•	11	M	Pre-T	1.57	5.4	48	5	25	20	4	40	No

•	5	M	Pre-T	3.63	11.2	70	40	43	10	5	40	No
•	10	F	Pre-T	3.05	10.4	60	36	35	10	4	40	No
•	6	M	Pre-T	4.01	13.9	14.1	38	30	-	-	65	No
•	4	F	Pre-T	1.55	4.6	37	58	84	5	5.4	60	No
•	2	M	Pre-T	3.46	7.1	55	33	20	10	14	35	No
•	3	M	Pre-T	4.34	8.8	102	55	76	25	4	50	No
•	3.5	M	Pre-T	3.29	12	34.3	62	10	29	3	55	Yes
•	4	M	Pre-T	2.4	4.5	153	10	38	10	9	86	No

Normal Range (Reference Values)

		CBC				**DLT**		
Age	**Sex**	**RBC** $\times 10^6$/ul	**Hg** g/dl	**WBC** $\times 10^3$/ul	**PLT** $\times 10^3$/ul	**L** %	**N** %	**M** %
<18	M	4.1-5.3	10.3-14.8	4.5-14.5	194-452	25-74	15-24	0-0.8
years	F	4.1-4.9	10.4-13.6	4.7-15.0	183-465			

CBC: Complete Blood Count, RBC: Red Blood Cells, Hg: Hemoglobin, WBC: White Blood Cells, PLT: Platelet, DLT: Differential Leukocyte Count, L: Lymphocyte, N: Neutrophil, M: Monocyte. * Normal Blast Cell percentage is less than 5% in the bone marrow.