Polymorphism of Interleukin-10 (IL-10, -1082 G/A) and Interleukin-28B (IL-28B, C/T) in pediatric Acute Lymphoblastic Leukemia (ALL)

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Abstract: We conducted genotypic analyses of interleukin-10 (IL-10) (-1082 G/A; GG, GA, AA) and interleukin-28B (CC, CT, TT) genes polymorphisms in acute lymphoblastic leukemia (ALL) pediatric patients in descriptive study to evaluate the prevalence of these mutations. In amplification refractory mutation system-PCR (ARMS-PCR), one reaction was carried out for each patient's DNA sample. For IL-28B gene, two forward and two reverse primers specific for C-allele and T-allele were used separately. For human IL-10 gene, two different forward primers specific for A and G alleles were used in combination with common reverse primer. IL-10 gene promoter showed highest frequency (n=29, 58%) of heterozygous (GA) allele, while genotypic analysis of IL-28B gene showed highest frequency (n=28, 56%) of homozygous (CC) allele. The IL-10 (AA) genotype related to its protein's less production in body which may be associated with the least survival of ALL's patients, while IL-28b (CT and TT) genotypes may be associated with less IFNλ3 levels and less life expectancy.

Keywords: Acute lymphoblastic leukemia (ALL); Interleukin 10 (IL-10); Interleukin 28B (IL-28B); Single nucleotide polymorphism (SNP)

INTRODUCTION

The prevalence of child acute lymphoblastic leukemia (ALL) varies from 0.9-4.7 per 100,000 children per year around the world (Zhang *et al*. 2011; Koju *et al*. 2015). In Pakistan, the mortality rate of child ALL is highest (71%) in the world (Ashraf, 2012). Interleukin-10 prevents inflammation during host immune response to infections and tumors through its anti-inflammation effects by inhibiting the synthesis of Th1 cytokine co-stimulatory molecules on macrophages and MHC class I and class II molecules' expression (Chen *et al*. 2013; Kong *et al*. 2010). In humans, the location of the IL-10 gene is at chromosome-1 consists of 5 exons and spans approximately of the size of 5.2 kb which encodes a homodimeric protein composed of two polypeptide chains of 178 amino acids (Mosser *et al*. 2008). Previous studies have reported biallelic polymorphism in the promoter region of IL-10 gene including 592 A/C (rs1800872), 819 T/C (rs1800871) and -1082 A/G (rs 1800896), which affects the transcription and translation processes of IL-10 *in vitro*.

High expression of IL-10 was observed in case of G-allele at position -1082 as compared to A-allele at the same position (Suarez *et al*. 2003). Patients with acute lymphoblastic leukemia (ALL) have much less survival time with genotype IL-10AA as compared to the genotype IL-10GG which presents a higher survival time (de Deus *et al*. 2012). Interleukin-28B with its two isoforms, i.e. IL-28A and IL-28B, is a small protein that regulates the immune system. In viral infections and tumors, the IL-28 acts as a potential therapeutic alternative to type-I IFN (Witte *et al*. 2010). In humans, its gene is located at chromosome-19 (19q13.13). Three different polymorphisms at three different positions near IL-28B gene including rs12980275A>G, rs12979860C>T and rs8099917T>G have been reported (Lee *et al*. 2013). High expression of IL-28B is seen in IL-28C-allele as compared to IL-28T-allele at the same position. Patients with IL-28B genotype having alleles-CT & TT present less survival time as compared to the genotype allele-CC which has a much better survival time (Akkiz *et al*. 2014). An international project had identified following fusion genes: *BCR-ABL1, ETV6-RUNX1, TCF3-PBX1* and *MLL-AF4* in children of developing countries (India, Pakistan, Myanmar, and Sudan) for additional information on prognosis (Siddiqui *et al*. 2010). The current research was conducted to investigate human interleukin 10 (IL-10, -1082 G/A) and human interleukin 28b (IL-28B C/T) polymorphisms simultaneously in acute lymphoblastic leukemia (ALL) child patients for the first time in

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Pakistan. The association between single nucleotide polymorphisms (IL-10, -1082 G/A & IL-28B, C/T) with acute lymphoblastic leukemia (ALL) was analysed in pediatric ALL patients. The frequencies of these single nucleotide polymorphisms were calculated.

MATERIALS AND METHODS

Study settings

The study was performed from June, 2015 to March, 2016. The blood samples were obtained from the Department of Hematology and Oncology, Children Hospital and Institute of Child Health, Lahore. It was a descriptive study. DNA extraction and molecular analyses were performed at Department of Allied Health Sciences & Chemical Pathology and Department of Human Genetics & Molecular Biology, University of Health Sciences (UHS), Lahore. The Lahore (capital of province Punjab) city is located in northwest (31.5204° N, 74.3587° E) of Pakistan. The current population is 11,126,285 950 according to the survey in 2017. This study was conformed to institutional ethical standards.

Study population

Fifty pediatric patients (n=50) from peripheral areas of Lahore, were diagnosed with acute lymphoblastic leukemia (ALL) and enrolled according to the inclusion and exclusion criteria (see below) for the molecular analysis of two human interleukin genes to assess the presence and prevalence of IL-10 -1082 G/A (GG, GA, AA) and IL-28B C/T (CC, CT, TT) polymorphisms. The patients visited to Department of Hematology and Oncology, Children Hospital and Institute of Child Health, Lahore from June, 2015 to March, 2016 were enrolled in current study.

Inclusion and exclusion criteria

We included newly diagnosed ALL cases which had not received blood transfusion and chemotherapy. ALL patients who have either received blood transfusion or on chemotherapy were not included in the current research.

Sample collection

Blood samples were collected from pediatric ALL patients (n=50) who were selected on the basis of inclusion and exclusion criteria. The informed consents were obtained from the attendants of children included in the current research. The appropriate amount of venous blood (1-3 ml) were collected by using 5 ml disposable syringes or 25G Butterflies. The blood samples were dispensed into EDTA containing blood collection tubes. The tubes were labeled with sample number. Each patient's serum and blood samples were stored in a freezer at -10 ^{0}C for subsequent molecular analysis.

Methodology

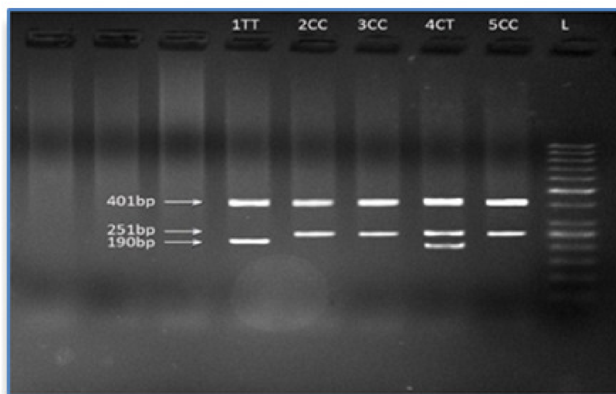
Molecular Analysis of IL-10 and IL-28B Genes

Molecular analyses of IL-10 (-1082 G/A) and IL-28B (C/T) polymorphism was conducted which involved the following procedure: The genomic DNA isolation from the ALL patients' blood samples was carried out by adopting the procedure of "salting out method" as described earlier (Miller *et al*. 1988) with some modifications. The patients' samples processed same day for DNA isolation and serum separation. The quality of extracted DNA was analyzed by "nano-drop" method. The polymorphism in IL-10 (-1082 G/A) was analyzed by allele specific PCR. In allele specific PCR, for human IL-10, two reactions were carried out simultaneously for each patient's DNA sample, one with a primer specific for G-allele and the second containing a primer for A-allele of the human IL-10 gene. In current research, for IL-10, two different forward primers specific for A-allele (1082A: 5'-CCTATCCCTACTTCCCCT-3') and G-allele (1082G: 5'-CCTATCCCTACTTCCCCC-3') of the human IL-10 gene (Accession no. NG_012088.1) were used in combination with a common reverse primer (R: 5'-AGCAACACTCCTCGTCGCAAC-3') as reported earlier (Settin *et al*. 2009; de Deus *et al*. 2012). For IL-28B, one reaction was carried out for each patient's DNA sample, with two forward and two reverse primers specific for C-allele and T- allele of the human IL-28B gene (Accession No.001276254.2). Two different forward primers (Forward inner: 5'-AGG GAG CTC CCC GAA GGA GT-3' and Forward outer: 5'-AAC TCA ACG CCT CTT CCT CCT-3') specific for C and T-alleles of the human IL-28B gene were used in combination with two reverse primers (Reverse inner: 5'-TGC AAT TCA ACC CTG GTG CG-3' and Reverse outer: 5'- TTC CCA TAC ACC CGT TCC TGT-3') as reported earlier (Galmozzi *et al*. 2011). PCR machine used was Bio-Rad Thermal cycler (Model T100). In all PCR reactions for IL10 and IL-28B, the concentration of all primers used was 100 picomole/µL, DNA was 300 nanogram/ µL, PCR buffer was 1X, $MgCl_2$ was 1.5mM, dNTPs was 0.2mM and Taq Polymerase was 2.5 U/reaction. For both genes, PCR temperature cycling condition was; initial denaturation at 95˚C for 4 minutes, cycling denaturation at 93˚C for 30 seconds, annealing at 61˚C for 1 minute, extension at 72 ˚C for 1 minute and final extension at 72 ˚C for 5 minutes. Reaction was terminated at 4˚C. In agarose gel electrophoresis, TAE (1X) buffer was used and 1.5% agarose gel electrophoresis was carried out and gel was run for 40 minutes at 100 volts for resolution of amplified PCR products.

Statistical analyses and graphical representations

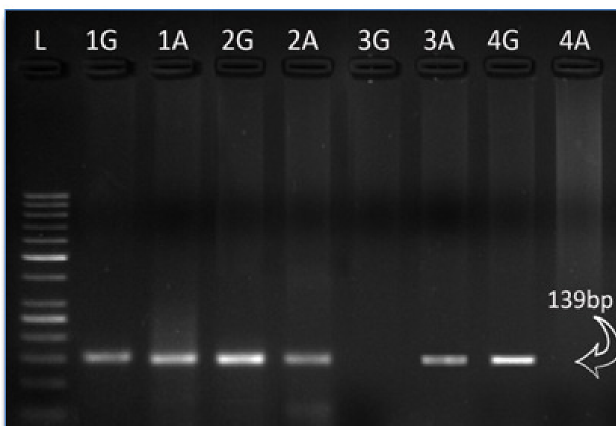
Data analysis was performed in SPSS (v.21.0, IBM Corporation) software. A Chi Square (χ^2) test was applied to look a relative significance at $p<0.050$ in the blood parameters between normal and disturbed values out of total. A Chi Square (χ^2) test was applied to look a relative significance at $p<0.050$ in the following gene alleles: GA, AA & GG of human IL-10 out of total. A Chi Square (χ^2) test was applied to look a relative significance at $p<0.050$ in the following gene alleles: CC, CT & TT of human IL-

28B out of total. The Box-whisker plot was generated in a software 'R' (64-bit) version (3.2.3) to compare medians, inter-quartiles, centralities etc. of IL-10 G/A and IL-28B C/T frequencies occurred in ALL patients.



Legends: L= Ladder DNA; 1TT= T-allele, 2CC= C-allele, 3CC=C-allele, 4CT= CT allele, 5CC= C-allele

Fig. 1: Representating photograph showing different alleles of human interlukin 28b (IL-28b) gene in ALL patients. (Cat No. SM0373, Thermoscientific, USA).



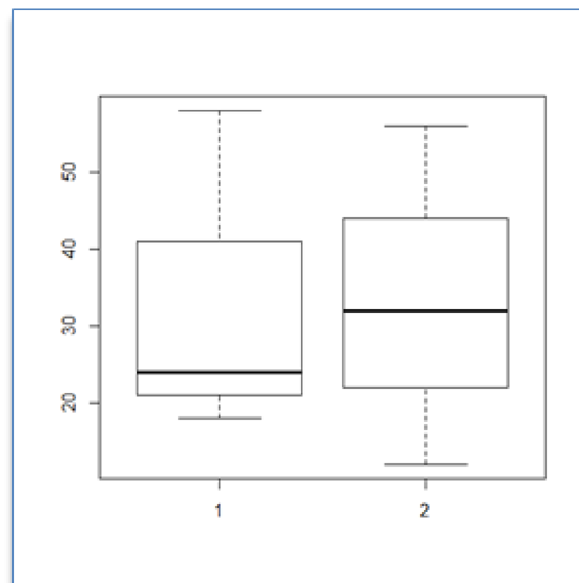
Legends: L= DNA Ladder; 1G=G-allele, 1A= A-allele, 2G=G-allele, 2A=A-allele, 3G= G-allele, 4G= G-allele, 4A= A-allele

Fig. 2: Representative photograph showing amplified products of Allele A and Allele G of IL-10 gene in ALL patients. Key: 1-4 patients samples checked for both A and G alleles. (Marker, Cat # SM0373, Thermoscientific, USA)

RESULTS

Polymorphism of IL-10 and IL-28B

The PCR amplified products of DNA samples of ALL patients having size of 139 bp representing A-allele and G-allele of the human IL-28b gene (fig. 1), while 251bp for T-allele and 190bp for C-allele of the IL-10 gene (fig. 2). The amplified PCR products sizes were analyzed on 2% agarose gel by visualizing the gel in UV transilluminator. From molecular analysis results it was observed that IL-10 gene promoter region (-1082 G/A) showed the highest frequency of heterozygous GA-allele was found in 29 (58%) of patients, homozygous AA-allele was found in 12 (24%) of patients and homozygous GG-allele was found in the remaining 9 (18%) of patients out of 50 enrolled ALL patients. The genotypic analysis of IL-28B gene showed the highest frequency of homozygous CC-allele that was found in 28 (56%) of patients, heterozygous CT-allele was found in 16 (32%) of patients and homozygous TT-allele showed least prevalence 6 (12%) out of 50 enrolled ALL patients as shown in table 1.



Legends: Horizontal axis: Box 1: IL-10 G/A patient group; Box 2: IL-28B patient group. Vertical axis: IL-10 G/A and IL-28B C/T frequencies. Box plot 1: max: 58; min: 18; median: 24; q1 (quartile 1):19.5; q3:49.5. Boxplot 2: max: 56; min: 12; median: 32; q1 (quartile 1): 17; q3: 50

Fig. 3: Box-Whisker Plot-A graphical comparison between IL-10 G/A polymorphism and IL-28B C/T polymorphism in ALL patients.

Chi square (χ^2) test and box whisker plots

There existed a significant difference (p=0.001) in the following gene alleles: GA, AA & GG of human IL-10 (χ^2=13.960; df=1) out of total. There existed a significant difference (p=0.001) in the following gene alleles: CC, CT & TT of human IL-28B (χ^2=14.560; df=1) out of total. fig. 3 shows a box whisker plot created for the comparison of IL-10 G/A and IL-28B C/T frequencies in ALL patients. Boxplot-1 is for the group IL-10 G/A polymorphism, whereas, boxplot-2 is for the group IL-28B C/T polymorphism. There is a little difference between two boxes' lengths (inter-quartile range). The centrality of two group boxes does not match, although, there is somehow an equal spread of the boxes. In boxplot-1, the top whisker is much longer than the bottom whisker unlike in the boxplot-2 where the symmetry can be seen in its tail-length.

Table 1: Frequency distribution of human IL-10 and IL-28b gene alleles in pediatric ALL patients (n=50)

Type of parameter		No. of patients	Percentage Prevalence (%)	*p*-value	**df*	*Chi-square* χ^2
IL-10 G/A	*GA*	29	58	0.001**	2	13.960
	AA	12	24			
	GG	9	18			
IL-28B C/T	*CC*	28	56	0.001**	2	14.560
	CT	16	32			
	TT	6	12			

*degrees of freedom, **significant ($p<0.05$)

DISCUSSION

The current research was conducted to find out frequency of single nucleotide polymorphisms in IL-10 (-1082 G/A) and IL-28B (C/T) genes which play important roles in human immune system. From genotypic analysis, it was observed that IL-10 gene promoter (-1082) region had the highest frequency (58%) of heterozygous (GA) allele in ALL patients while the frequency of homozygous allele (AA) and (GG) was found to be 24% and 18% respectively. The genotypic analysis of IL-28B gene showed the highest frequency (56%) for homozygous (CC) allele in patients whereas, the frequency of heterozygous (CT) allele and homozygous (TT) allele was found to be 32% and 12% in ALL patients. Overall, heterozygous (GA) allele and homozygous (CC) allele for IL-10 and IL-28B genes respectively were dominated in pediatric ALL patients.

It was reported that IL-10 high expression was observed for G-allele at position -1082 as compared to A-allele at the same position (Suárez *et al*. 2003). It was also mentioned that ALL patients with genotype (AA) have much less survival time as compared to (GG) genotype which presents a higher survival time (de Deus *et al*. 2012). Therefore, based on findings of previous research, in the current study heterozygous (GA) allele for IL-10 gene promoter (-1082) region was observed in 58% ALL patients and these patients may have high IL-10 expression level and high survival time. The IL-28b (CT and TT) genotypes may be associated with less IFNλ3 levels and less life expectancy. In the current research highest frequency (56%) of a normal CC-allele of IL-28B was observed in ALL patients suggesting that this allele is dominant in local population of ALL patients. A study by De dues *et al*. (2012) had reported IL-10 homozygous allele-AA in 25% of patients, homozygous allele-GG in 27% of patients, while, 48% were found to be in heterozygous-AG state. Afzal *et al*. (2011) conducted a research to find out the relation of IL-10, -1082 G/A polymorphism in Hepatitis-C patients in Pakistani population and showed prevalence of 8% of AA-allele, 17% of GG-allele and 75% AG-allele. A research in Taiwan concluded that genotypes of IL-10-819C and A-592C can be considered as biomarkers for the diagnosis of pediatric ALL (Lo *et al*. 2016).

The current research can be applied to relatively high number of ALL patients as time and resources availability were main limiting factors in the current research.

CONCLUSION

The findings of current research suggested that IL-10 gene promoter region (-1082) with (GA) genotype and IL-28B gene with (CC) genotypes are most prevalent in ALL patients. IL-10 (-1082, AA and GG) genotypes have prevalence less than 25% in ALL patients while for IL-28B, genotypes (CT, TT) have prevalence less than 33% in ALL patients.

Compliance with Ethical Standards Statement: Informed consents were obtained from all patients included in the study conformed to institutional ethical standards. The author reports no conflict of interest.

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