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Polymorphism of CD4 and CD8 gene and soluble HLA-G as novel markers in acute lymphoblastic leukemia patients

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Abstract

"Acute lymphoblastic leukemia is a malignant proliferation of lymphoid cells blocked at an early stage of differentiation". CD4 and CD8 genes encode a membrane glycoprotein of T lymphocytes that interact with MHC class I and II antigens. Soluble HLA-G is an immunosuppressive molecule that induces activated CD8 T cell apoptosis and down-modulates the proliferation of CD4 T cells. The major aim of this study is to assess the genetic polymorphism of the human CD4 and CD8 molecules in a acute lymphoblastic leukemia (ALL) patients and to determine if polymorphisms in either CD4 or CD8 and soluble HLA-G are related to the susceptibility of ALL. The CD4 and CD8 genes were successfully amplified using specific PCR primers (primer design). Results of CD4 and CD8 gene were an amplified product at 285 bp and 517bp. DNA sequencing showed there were two polymorphisms in CD8 gene: First polymorphism (Transversion) leading to change of cytocine to guanine (G>C, 529), and second polymorphism (Transversion) leading to change of adenine to cytosine (C>A,230). While no polymorphism of the CD4 gene was found. The mean of soluble HLA-G levels in ALL patients was significantly increased 60.3±38.12ng/ml, compared with the healthy control group which was 12.18 ± 10.22 ng/ml. Conclusion: polymorphism of the CD8 gene and sHLA-G can be considered as a good biomarker for the diagnosis and/or prognosis of ALL.

Keywords: Acute lymphoblastic leukemia, CD4 and CD genes, Polymorphism , Human leukocyte antigen-G

INTRODUCTION

Acute lymphoblastic leukemia (ALL) is a malignant lymphatic cell proliferation blocked at an early stage of differentiation. ALL is a biologically heterogeneous disease, so that morphologic, cytogenetic, biochemical, immunologic and molecular genetic characterization of leukemic lymphoblasts are required for the confirmation of the diagnosis or for exclusion of other causes of bone marrow failure and, finally, for classification of ALL subtypes [1]. Glycoproteins of T cell surface (T4 & T8) are believed to mediate effective cell-cell interactions in the immune system and they may be responsible for the suitable of T cell subpopulation targeting (Littman et al, 1985). CD4 gene encodes the membrane T-cell glycoprotein that interacts with class II antigens of the major histocompatibility complex (MHC), and is also the human immunodeficiency virus (HIV) receptor. This gene is expressed not only in T-cell lymphocytes, but also in other cells like B-cells, granulocytes and macrophages, as well as some specific parts of the brain. In this gene, multiple alternatively spliced transcript variants encoding different isoforms were recognized. The CD8 antigen is a cell surface glycoprotein present on most cytotoxic T –cells which mediates effective cell-cell interaction in the immune system [2]. The CD8 antigens work as co-receptors with the T-cell receptors on the T- cell lymphocytes for the purpose of recognition of antigens displayed on antigen presenting cells (APCs) in the context of MHC molecules class I. The function of the co-receptors is either as homodimers consisting of two alpha chains or as heterodimers consisting of one alpha and one beta chains. Significant homologies to immunoglobulin variable light chains are shared by both the alpha and beta chains. The CD8 alpha chain is encoded by this gene, and for this gene, multiple transcript variants encoding different isoforms were detected [3]. In addition, the human leukocyte antigen-G or (HLA-G) is different from other MHC class I genes by its low polymorphism and by alternative splicing that generates (7) HLA-G proteins, whose tissue distribution is confined to normal adult and fetal tissue which displays a tolerogenic function towards both acquired and innate immune system cells. Soluble HLA-G is the immunosuppressive molecule that induces apoptosis of activated CD8(+) T-lymphocyte and down-modulates CD4(+) T-lymphocyte proliferation [4]. HLA-G is believed to be the mechanism of tumor-driven immune escape [4], thereby protecting cancer cells from the destruction by NK and cytotoxic T cell-mediated cells [5]. In this context, HLA-G expression may be a mechanism used by cancer cells to escape host immune surveillance [6]. The blocking of HLA-G at the expression level (by RNA interference) or function (by neutralizing antibodies) may promote tumor cell clearance by the immune system [5]. However, for patients with leukemia, there is always limited data, and conclusions remain controversial[7]. There are two main aims: a) to evaluate the genetic polymorphism of the human CD4 and CD8 genes in acute lymphoblastic leukemia patients, and study novel marker sHLA-G that are related to the susceptibility of ALL.

MATERIALS AND METHODS

Whole blood samples were obtained from 50 patients affected with acute lymphoblastic leukemia whose ages ranged from 4 to 25 years and admitted to the National Center of Hematology/ Al Mustanisyria University. Blood samples were also taken from (25) healthy subjects as a control group for comparison. The disease was clinically diagnosed by the consultant medical staff at the centre. DNA was extracted from the samples by the kit of DNA extraction from (Wizard® Genomic DNA Purification Kit, Promega, Madison, WI, USA) pursuant to the manufacturer's instructions for isolating genomic DNA from whole blood samples.

Molecular detection

Primer design

The digested DNA was subjected to PCR amplification with the designed primers by using Primer 3 plus Software from (SourceForge, Inc., Mountain View, CA, USA). Detection of *CD8* gene was conducted by using primers for amplification of for *CD8* gene. The fragment 517 bp of *CD8* was amplified using a forward and reverse primers respectively (CD8F:5′-AGCGACCATCATTGTAGCCA3′)and(CD8R:5′(GAGAGTGCA GACATGACGCT-3′), and the fragment 285 bp of *CD4* was amplified using a forward and reverse primers respectively (CD4F: 5′-AGCGACCATCATTGTAGCCA-3′) and (CD4R:5′-GAGAGTGCAGACATGACGCT-3′) (Primers set provided by alpha DNA Company, Canada).

Polymerase chain reaction

The PCR was amplified in a 25µl volume which contains (1.5µl) DNA, (12.5 µl) Go Taq green master mix 2X from (Promega corporation, USA), and (1µl) of each primer (10 Pmol), then (25µl) of nucleases free-water was added to complete the volume. The following steps were done to perform thermal cycling

conditions: Denaturation for (7) minutes at (94 °C), followed by (35) cycles for (30) seconds at (94 °C), (1) minute at (63°C), and (30) seconds at (72°C) with a final incubation for (5) minutes at (72°C) by using the thermal cycler from (Gene Amp, PCR system 9700; Applied Biosystem). The (1.5%) agarose gel electrophoresis was used for PCR product separation, and the exposure to ultraviolet light (302)nm was used for its visualization after the staining by ethidium bromide. The sequencing and purification of PCR products in both directions was done by the sequencing kit (BigDye Terminator v3.1 Cycle on an ABI 3130 Genetic Analyser Applied Biosystems, Foster City, CA). CD4 & CD8 genes were done by Macro gen company for product sequencing, Korea . Homology search was done by the program of sequence of standard gene BLAST, available at the national center biotechnology information (NCBI) online at (http:// www.ncbi.nlm.nih.gov) and by applying the BioEdit program.

Estimation of soluble HLA-G in serum samples

From 50 ALL patients and 25 healthy control subjects, 3 ml of blood was collected and centrifuged to obtain serum. Serum soluble HLA-G was estimated by ELISA kit according to the kit protocol. This kit uses the double antibody sandwich technique to measure serum concentrations of soluble HLA-G (HANGZHOU EASTBIOPHARM, China)

RESULTS

The specific PCR primer (primer design) was successfully used to amplify *CD4 and CD8* genes. Figure (1) showed the *CD4* and *CD8* where a specific product at 285 bp and 517bp was identified.

Sequencing of CD4 and CD8 genes

Sequencing of CD4 and CD8 genes was performed to detect polymorphism of these genes among 50 patients with acute lymphoblastic leukemia and 25 healthy control group. All results were compared with the human reference sequence (http: NCBI Reference Sequence) by a software program (BioEdit program) available in the website.

The results of DNA sequencing were summarized in table (1) which showed there were two polymorphisms in CD8 gene: First polymorphism (Transversion) leading to change of cytosine to (**G>C**, 529) and second polymorphism (Transversion) leading to change of adenine to cytosine (**C>A**,230) (figure 2). While no polymorphism in CD4 gene (figure 3).

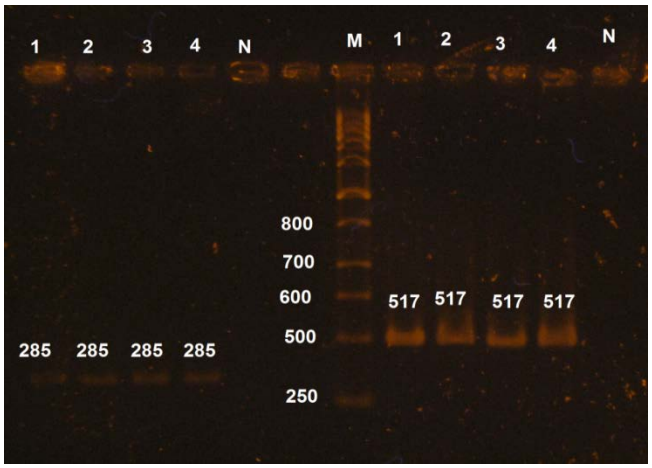

Figure 1: Agarose gel electrophoresis for *CD4* & *CD8* lymphocyte gene amplification belonging to healthy and ALL patients was done. Electrophoresis was applied for band fractioning on (1.5%) agarose gel (2 h., 5V/cm, 1X Tris-acetic buffer) and U.V light was applied for their visualization after staining with ethidium bromide staining. Lane M:250bp ladder. Lane:1, (Healthy), Lane:2,3,4 (ALL).

Homo sapiens CD4 molecule (CD4), RefSeqGene on chromosome 12, Sequence ID: ref|NG_027688.1|

Score	Expect	Identities	Gaps	Strand
523 bits(283)	3e-145	283/283(100%)	0/283(0%)	Plus/Plus

```
Query  1     GTGGGGCAGATGCACAGGAATGTGCTCTGCCCAGTTGTCTGCCCACAGCTCTGGCCACCT  60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  5243  GTGGGGCAGATGCACAGGAATGTGCTCTGCCCAGTTGTCTGCCCACAGCTCTGGCCACCT  5302

Query  61    TCTCTTGCATTTCTCTTGGAACTGGTCATGAGCAGCGATTTCCCACTGGAACTGTGAGCT  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  5303  TCTCTTGCATTTCTCTTGGAACTGGTCATGAGCAGCGATTTCCCACTGGAACTGTGAGCT  5362

Query  121   TCCAGAGGTCAGAGACTGTGCTAGACTCCTCTCTGCAGCCCCAGCGTGCACAGCTCAGTG  180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  5363  TCCAGAGGTCAGAGACTGTGCTAGACTCCTCTCTGCAGCCCCAGCGTGCACAGCTCAGTG  5422

Query  181   TCCAGAGCAATGGGTGCTCCTTAGAGGAGTAGTGACCTAAATAGCAAGATCAGAGAGGGA  240
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  5423  TCCAGAGCAATGGGTGCTCCTTAGAGGAGTAGTGACCTAAATAGCAAGATCAGAGAGGGA  5482

Query  241   GTGAAGACTGGAGACTATCCCAGGCTGGGAAAGGCGTGGAAGG  283
             |||||||||||||||||||||||||||||||||||||||||||
Sbjct  5483  GTGAAGACTGGAGACTATCCCAGGCTGGGAAAGGCGTGGAAGG  5525
```

Figure 2: Alignment of *CD4* gene: BLASTn results for *CD4* gene showing 100% identity for the healthy control group as compared with the standard *CD4* obtained from Gene Bank.

Table 1: Types of polymorphism in *CD4 gene* compared with Homo sapiens CD8A intronic regulatory region, Ref Seq Gene/ chr 2.

Number of samples	Type of substitution	Location	Nucleotide	Range of nucleotide	Sequence ID	Score	Expect	Identities	Source
1	Transversion	529	G>C	64 to 535	ID: NG_044961.1	843	0.0	99%	Homo sapiens CD8A intronic
	Transversion	530	C>A						
2	----------			65 to 528	ID: NG_044961.1	838	0.0	100%	Homosapiens CD8A intronic

Homo sapiens CD8A intronic regulatory region on chromosome 2, Sequence ID: NG_044961.1.

Score	Expect	Identities	Gaps	Strand
843 bits(934)	0.0	470/472(99%)	0/472(0%)	Plus/Plus

```
Query  326  AGCTGACCTGATTCTCAGCCCCGGAGGATGACAGGGAGAGAGGAGGATGTGAGCAAATCA  385
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  364  AGCTGACCTGATTCTCAGCCCCGGAGGATGACAGGGAGAGAGGAGGATGTGAGCAAATCA  423

Query  386  CCACCATCAGCCAAATGATGTTACGCTAAAAACGTGTTAATTCAGCACCAGGCTAGCACC  445
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  424  CCACCATCAGCCAAATGATGTTACGCTAAAAACGTGTTAATTCAGCACCAGGCTAGCACC  483

Query  446  TTGTAAACATGCTAATTCATCATGAGCGTCATGTCTGCACTCTCACACCTCT  497
            |||||||||||||||||||||||||||||||||||  |||||
Sbjct  484  TTGTAAACATGCTAATTCATCATGAGCGTCATGTCTGCACTCTCAGCCCTCT  535
```

Figure (3): Alignment of *CD8* gene, BLASTn results for *CD8* gene showing 99% identity. for healthy control groups as compared with standard *CD8* obtained from Gene Bank.

Sequencing of *CD8* gene for acute lymphocyte leukemia patients as compared with standard *CD8* obtained from Gene Bank.

Homo sapiens CD8A intronic regulatory region (LOC106699567) on chromosome 2, Sequence ID: NG_044961.1

Score	Expect	Identities	Gaps	Strand
839 bits(930)	0.0	465/465(100%)	0/465(0%)	Plus/Plus

```
Query  324  TAGCTGACCTGATTCTCAGCCCCGGAGGATGACAGGGAGAGAGGAGGATGTGAGCAAATC  383
            |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  363  TAGCTGACCTGATTCTCAGCCCCGGAGGATGACAGGGAGAGAGGAGGATGTGAGCAAATC  422

Query  384  ACCACCATCAGCCAAATGATGTTACGCTAAAAACGTGTTAATTCAGCACCAGGCTAGCAC  443
            |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  423  ACCACCATCAGCCAAATGATGTTACGCTAAAAACGTGTTAATTCAGCACCAGGCTAGCAC  482

Query  444  CTTGTAAACATGCTAATTCATCATGAGCGTCATGTCTGCACTCTC  488
            |||||||||||||||||||||||||||||||||||||||||||
Sbjct  483  CTTGTAAACATGCTAATTCATCATGAGCGTCATGTCTGCACTCTC  527
```

Figure 4: Alignment of *CD8* gene: BLASTn results for *CD8* gene showing 100% identity for the healthy control group as compared with the standard *CD8* obtained from Gene Bank.

Measurement of sHLA-G in serum samples of ALL patients and the control group

The mean of soluble HLA-G levels of ALL was significantly increased (60.3±38.12) ng/ml, P < 0.001. While the mean of sHLA-G serum level of the healthy subjects was 12.18 ± 10.22 ng/ml, as shown in table 2.

Table 2: The Mean of s HLA-G levels in serum of the studied groups.

Subjects	Mean sHLA-G levels	P value
Acute lymphoblastic leukemia	60.3±38.12	0.001
Healthy control group	12.18 ± 10.22	

DISCUSSION

Cancer development is correlated with the host's immune suppression, which results in failure to produce an effective immune response against cancer cells [8]. The mechanism of specific T cell defects is not well understood, but it includes the production of immune-suppressive factors by cancer cells, direct tumor cell–T cell interaction, and induction of regulatory T cell subsets. In leukemia, there is cancer cell circulation, so there is a widespread interaction of tumor cells with T cells that can readily be obtained from the peripheral blood. The glycoproteins found on T cell surface (T4 & T8) are thought to mediate effective cell-cell interactions, and in this way, they may be responsible for the proper targeting of T cell subpopulations.

Our study is considered as the first trail to evaluate the genetic polymorphism of the human CD4 and CD8 genes in Iraqi patients with ALL by Primer Design for CD4 and CD8 genes. In the current study, two polymorphisms were found in CD8 gene: First, polymorphism (Transversion) leading to change of cytosine to guanine (G>C, 529) and second, polymorphism (Transversion)

leading to change of adenine to cytosine (C>A,230), while no polymorphism was found in CD4gene.

The sHLA-G in acute leukemia patients was estimated by Enzyme-Linked Immunosorbent Assay. Our results showed increased secretion of sHLA-G in ALL patients compared with the control group. These results were compatible with the study of Gros et al who found elevated sHLA-G in subtypes of acute leukemia subtypes that affect lymphoid and monocytic lineages like FABM4 and FABM5, in addition to both T and B acute lymphoblastic leukemia (ALL) [10].Another study found a statistically significant increase in the sHLA-G level in AML patients compared with the control group, and being higher in relapsed cases[11].

A statistically significant difference was found by Locafaro et al between sHLAG levels in new and relapsed cases of AML compared with the control group ($p=0.001$), when they detected higher sHLAG levels in relapsed patients than in new AML cases ($p < 0.05$). Results in our study revealed that 80% (24/30) of AML subjects had higher than the cutoff point (10/24) were newly diagnosed AML and (14/24) were relapsed AML cases[12]. The variation of results may be associated with the different ethnic population studied. The discrepancy may also be attributed to the higher sHLA-G (shed HLAG1 and HLA-G5) in EDTA-treated plasma compared to the heparin-treated plasma heparin or serum.

The elevated sHLA-G levels can be associated with 2 biologic features: High-level leukocytosis and absence of myelodysplasia. In acute leukemia, myelodysplasia is one of the factors that are related to prognosis and, is nowadays involved in the classification of the World Health Organization [10].

Several studies on human leukocyte antigen-G expression in leukemic patients provided discordant conclusions. The results of other researchers found no membrane-bound or cytoplasmic HLA-G expression. Soluble HLA-G isoforms seem to be more frequent than membrane-bound isoforms in hematopoietic malignancies such as lymphoproliferative disorders [13][14].

sHLA-G molecules seem more frequently expressed than membrane-bound isoforms during hematologic malignancies, such as lymphoproliferative disorders. The present findings indicated that sHLA-G molecules might be a factor in the escape of tumor cells from the immune surveying during acute leukemias. In acute leukemias, levels of soluble HLA-G molecules are elevated, especially in the subtypes that affect lymphoid and monocytic lineages[15].

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