Detection of mutation in Exon 2-3 in Perforin gene and Exon4 in Fas gene in sample leukemia Iraqi patients

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ABSTRACT

This work aimed to study the possible mutations in Perforin gene (PRF1) exon 2-3 and Fas gene exon 4 in Acute Lymphocytic Leukemia (ALL) and Chronic Lymphocytic Leukemia (CLL) patients from Iraq. In an attempt to detect any mutation within PRF1 gene and Fas genes, a sequencing analysis for these genes were made. The results were alignment with sequences present in the Gene Bank seeking for homology and differences. A DNA sequence for Homo sapiens PRF1 gene was found compatible with genes of ALL, CLL patients and healthy controls, 100% compatibility was found in the flank DNA sense and antisense sequences from healthy. However, 99% compatibility was detected for the genes isolated from ALL patients with an insertion of C697 and A698 G in the flank DNA sense strand and insertion of G697 and T698C in flank DNA antisense strand of the gene. Moreover, 99% compatibility was detected for the genes isolated from CLL patients with two transition mutations in the flank DNA sense strand of C957T and C1035T and one transition mutation in the flank DNA antisense strand of G957A. However, no mutations were detected in Fas gene isolated from ALL, CLL, and healthy controls.

INTRODUCTION

In humans, perforin deficiency leads to a potentially fatal disorder in infancy, familial hemophagocytic lymphohistiocytosis type 2 (FHLH2) (1). Patients with mutations in the perforin gene (PRF1) have absent or low perforin levels in NK cells and diminished lymphocyte cytotoxicity (2). Missense mutations in PRF1 have also been described in an adult with chronic active epstein barr virus (EBV) infection (3) and in children with bone marrow malignancies (4). The phenotypic expression of PRF1 mutations is variable, and that the spectrum of perforin-related disease may include fatal immune dysregulation in early childhood; nonfatal, inflammatory reactions at any age and impaired tumour surveillance in children and adults. In other studies of FHLH2, over 50 mutations of the perforin gene (1 & 5) have been identified, most of the perforin mutations in patients with FHLH2 do not lead to severe protein truncation but consist of amino acid substitutions and detection of mutant perforin by Western blotting of perforin lysates from individuals (6). Missense mutations in perforin, a critical effector of lymphocyte cytotoxicity, lead to a spectrum of diseases, from FHLH2 to an increased risk of tumorigenesis (1). Most missense PRF1 mutations in FHLH2 patients result in loss of function of perforin, most commonly due to unfolding and faulty trafficking of the protein (7 & 8), the mutation identified in perforin result in loss of a functional mRNA and complete loss of perforin protein or non functional protein (9 & 10). The present study aimed to investigate correlation between mutation in PRF1 and Fas gen and increasing leukemia in Iraqi population.

Materials and methods
Collection of samples

Five ml of blood was collected by vein puncture from 39 cases (21 ALL and 18 CLL) who were admitted to the National Center of Haematology/ Al Mustansiriyah University. The disease was clinically diagnosed by the consultant medical staff at the centre. In addition, 5 apparently healthy controls (blood donors) were also included.

Isolation of Lymphocyte

Preparation of solutions and media were done according to the methods described by [{11}, {12}] unless mentioned. The lymphocytes were isolated from the heparinized whole blood using the method described by [{13}] as follows: three ml of blood was centrifuged at 1000rpm for 15min. The plasma was collected for perforin estimation, buffy coat was collected in a 10 ml centrifuge tubes and diluted with 5ml RPMI 1640 (cell suspension), five ml of the diluted cell suspension was layered on 3ml of ficoll-isopaque separation fluid, the tubes were centrifuged at 2000rpm for 30min in a cooled centrifuge at 4°C. After centrifugation, the mononuclear cells were visible as cloudy band between the RPMI1640 and lymphoprep layers. The band was collected in a 10ml test tube and the cells were suspended in 5 ml RPMI 1640. The tube was centrifuged at 2000rpm for 5min (first wash), the supernatant was discarded and the cells were resuspended in 5 ml RPMI 1640 (repeated twice). The suspension was centrifuged at 1000rpm for 10min, the supernatant was discarded. The precipitated cells were resuspended in 1ml RPMI solution and used in the planned experiments. Counting the cells were performed before experiment according to [{13}], the numbers of lymphocytes were counted by light microscope and the cells concentration was adjusted to 1X106 cell/ml. The isolated cells were grown in a flask containing 10ml RPMI 1640 medium supplemented with BSA and incubated at 37°C for 48h.

Isolation of Genomic DNA

Genomic DNA was isolated from culture cells under aseptic condition according to the protocol described by promega company for wizard genomic DNA purification kit (Cat #: A1120). Cells grown as liquid culture pellets were resuspended in PBS and vortex mixed. Nuclei lysis solution 600µl was added to cells grown as liquid culture and mixed by pipetting. RNase solution 3µl was added to the lysis solution 600µl was added to cells grown as liquid culture. The genomic DNA from 39 patient were extracted using Genomic DNA were amplified by using specific PCR primers for exon 2-3 gene (Exon 2-3) of lymphocyte belonging to healthy, ALL, and CLL patients was done. Bands were fractionated by electrophoresis on a 1.5 % agarose gel (2 h., 5V/cm, 1X Tris-acetic buffer) and visualized under U.V. light after staining with ethidium bromide staining. Lane M:100bp ladder. Lane:1.(Healthy), Lane:2.(ALL), Lane:3.(CLL).

Results and discussion

Amplification of Exon 2-3 Perforin Gene

The genomic DNA from 39 patient were extracted using wizard genomic DNA promega, PRF1 gene from genomic DNA were amplified by using specific PCR primers for exon 2-3, results shown in figure (1) indicated that a yield of single band of the desired product with a molecular weight about 572 bp for exon 2-3 gene was obtained.
Sequencing of coding regions of the amplified product (Exon 2-3) for these samples were done seeking for detection of any mutation within these sequence related to cancer development. Alignment of PRF1 gene of all groups (Healthy, ALL, and CLL) with data published for known sequence seeking for enough homology. A homology with PRF1 gene of Homo sapiens from the Gene Bank was done using the BioEdit software. 100% compatibility of that gene was found with PRF1 gene from all DNA sense and antisense of the gene) from healthy with standard PRF1 of Gene Bank results as shown in figure (2).

A: Sense of the partial PRF1 gene.

Score = 750 bits (406), Expect = 0.0, Identities = 406/406

B: Antisense of the partial PRF1 gene

Score = 689 bits (373), Expect = 0.0, Identities = 373/373

Figure (2): Sequencing of sense and antisense flanking the PRF1 gene for healthy as compared with standard PRF1 obtained from Gene Bank (A: Sense of the gene; B: Antisense of the gene).

The PRF1 gene from ALL patients showed 99% compatibility with standard PRF1 of Gene Bank, and there was insertion of C 697 and A 698 G in the flank DNA sense and insertion of G 697 and T698 C in flank DNA antisense strand leading to change all codons (frameshift mutation).

This can change amino acid and the effect could impair the function of perforin and cause problems in metabolic activity and effect function as shown in figure (3).

A: Sense of the partial PRF1 gene.

Score = 750 bits (406), Expect = 0.0, Identities = 411/413

B: Antisense of the partial PRF1 gene.
Figure (3): Sequencing of sense and antisense flanking the PRF1 gene for ALL patient as compared with standard PRF1 obtained from Gene Bank (A: Sense of the gene; B: Antisense of the gene).

The PRF1 gene from CLL patients showed 99% compatibility with standard PRF1 of Gene Bank. There were two transition mutations in the flank DNA sense strand C957 T and C1035 T while there are one transition mutation in the flank DNA antisense strand G 957 A as shown in figure (4); table (1).

A: Sense of the partial PRF1 gene.

Score = 739 bits (400), Expect = 0.0, Identities = 404/406 (99%), Gaps = 0/406 (0%), Strand=Plus/Plus

B: Antisense of the partial PRF1 gene.

Score = 684 bits (370), Expect = 0.0, Identities = 372/373 (99%), Gaps = 0/373 (0%), Strand=Plus/Minus
obtained from Gene Bank (A: Sense of the gene; B: Antisense of the gene).

Table (1): Types of mutations detected in partial PRF1 gene of CLL patients.

Human perforin gene mutations were detected previously by several investigators. For instance, nine types of nonsense mutations and other four types of missense mutations were characterized in three families of Japanese patients suffering from familial hemophagocytic lymphohistiocytosis FHLH2 (15). Also mutations in 12 base pair (codon 284-287) which are responsible for the change in four amino acids of the complex domain of a membrane protein were detected from Omani boy diagnosed at 44 days after birth suffering from FHLH2 disease (16). Later, many mutations were detected in the PRF1 gene from eleven patients (six males and five females) during DNA sequencing of exon 2 and exon 3, of these mutations (17), seven different changes were identified in the coding region of the perforin gene. Five of them (265C>A, 518C>T, 363C>T, 674 G>C and deletion 12 bp) are novel along with other (50 deletion T and 1122G>A). During this review, they recognized 40 mutations within coding region of PRF1 gene in different ethnic groups, but seven different mutations in the PRF1 gene in Omanis determined clinically to have FHLH2 with a family history. (5) referred to perforin mutation identified in 7 of the 34 families FHLH2 of Turkey investigated, six children were homozygous for the mutations and one patient was a compound heterozygote, four novel mutation were detected (one nonsense, two missense, and one deletion of one amino acid). On the other hand, referred to a mutation in exon 2 (del207C) and exon 3 (del 1009-91CT) were detected in FHLH2 patient from Japan lead to lower expression of perforin from lymphocyte of the patient (18). Moreover, three heterozygous mutations were detected in a coding region of perforin gene in three patients of hemophagocytic lymphohistiocytosis (14). While, 21 missense mutations in perforin gene of hemophagocytic lymphohistiocytosis patients lead to absent or low levels of perforin in NK cells (20).

During a study on 60 cases familial hemophagocytic lymphohistiocytosis (FHLH2) 22 missense mutations were detected (P39H, G45E, V50M, D70V, C73R, W95R, G149S, F157V, V183G, G220S, T221I, H222R, H222Q, I223D, R232C, R232H, E261K, C279Y, R299C, D313V, R361W and A523D). Other mutations were also detected by others investigator that reduced the functional activity and perforin expression such as A91V mutation in NK and CD8+ cells (21) and frame shift mutation in perforin gene leading to stop codon which cause loss of perforin functional activity (10).

Amplification and Sequencing of Partial Fas Genes

Fas gene from genomic DNA were amplified by using specific PCR primers for exon 1, results shown in figure (5) indicated that a yield of single band of the desired product with a molecular weight about 272 bp for exon 1 gene was obtained.

Figure (5): Agarose gel electrophoresis for amplified Fas gene (Exon 4) of lymphocyte belongs to healthy, ALL, and CLL patients. Bands were fractionated by electrophoresis on a 1.5 % agarose gel (2 h, 5V/cm, 1X Tris-acetic buffer) and visualized under U.V. light after staining with ethidium bromide. Lane 1 (Healthy), Lane 2 (ALL), Lane 3 (CLL).

After alignment of Fas gene of the healthy, ALL and CLL groups with the Fas of Homo sapiens from the Gene Bank using the BioEdit software, we found that part of Fas gene (Flank DNA sense and antisense of the gene) from healthy having 100% compatibility with standard Fas gene obtained from Gene Bank as shown in figure (6).

Figure (6): Sequencing of sense and antisense flanking the Fas gene obtained. (flank DNA sense and antisense of the gene) from healthy, ALL, and CLL patients. Bands were fractionated by electrophoresis on a 1.5 % agarose gel (2 h, 5V/cm, 1X Tris-acetic buffer) and visualized under U.V. light after staining with ethidium bromide. Lane 1 (Healthy), Lane 2 (ALL), Lane 3 (CLL).

A: Sense of the partial Fas gene.

Score = 204 bits (110), Expect = 2e-50, Identities = 110/110 (100%), Gaps = 0/110 (0%), Strand=Plus/Minus.

B: Antisense of the partial Fas gene.

Score = 204 bits (110), Expect = 9e-52, Identities = 110/110 (100%), Gaps = 0/110 (0%), Strand=Plus/Plus.
from Gene Bank (A: Sense of the gene; B: Antisense of the gene).

Moreover, we also found that Fas gene (flank DNA sense and antisense of the gene) ALL and CLL obtained from patients having 100% compatibility with standard Fas gene of Gene Bank as shown in figure (7).

A: Sense of the partial Fas gene.
Score = 204 bits (110), Expect = 2e-50, Identities = 110/110 (100%), Gaps = 0/110 (0%), Strand=Plus.

B: Antisense of the partial Fas gene.
Score = 204 bits (110), Expect = 9e-52, Identities = 110/110 (100%), Gaps = 0/110 (0%), Strand=Plus.

Figure (7): Sequencing of sense and antisense flanking the Fas gene (Exon4) for ALL and CLL as compared with standard Fas obtained from Gene Bank (A: Senses of the gene; B: Antisense of the gene).

Although the results did not detect any mutations in exon 4 of Fas gene, other investigators have detected a lack of 20 base pair at Exon 9 resulting in a frame shift mutation which resulting the generation of a pre mature stop codon at amino acid 239 of Acute T-cell leukemia (ATL) (22 & 23), specify deletions in exon 9 in Fas gene, five missense mutations and one silent mutation in all 65 human non small cell lung cancers using PCR and DNA sequencing, they found that changes lead to loss of cells apoptotic functions and contribute to the pathogenesis of some human lung cancer. A novel Fas mutation which predicted the truncation of the intracytoplasmic domain of the Fas receptor in two siblings and the loss of Fas antigen expression by skipping of exon 4 of the Japanese patients (lymphoproliferative disorder) were detected (24) and Point mutation that was present in the splice acceptor site of intron 3 of the Fas gene were detected previously (18), this mutation results in the skipping of exon 4 and the complete loss of Fas expression.

References


