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AUTOIMMUNE HEMOLITYC ANEMIA IN PATIENTS WITH CHRONIC LYMPHOCYTIC LEUKEMIA: A SINGLE CENTER EXPERIENCE

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Abstract

Autoimmune phenomena are a well-known complication of lymphoproliferative diseases, particular in chronic lymphocytic leukemia (CLL). Autoimmune hemolytic anemia (AIHA) is the most frequent autoimmune disorder described in CLL patients with unfavorable biological risk factors.

Aim of the study: Evaluation of AIHA in chronic lymphocytic leukemia patients from the Republic of North Macedonia in correlation with genetic structure of pathologic B lymphocyte.

Material and methods: This is a retrospective study of 100 patients with CLL, diagnosed and observed in the period between January 2012 and January 2022. Traditional laboratory, clinical and biological prognostic factors were evaluated at first patient visit to University Clinic of Hematology - Skopje Macedonia. Immunohematology analyses was performed at the Institute for Transfusion, Skopje, North Macedonia. Mutational status and configuration of IGHV-IGHD-IGHJ rearrangements and genetics were analyzed using reverse transcriptase– polymerase chain reaction (RT-PCR) and sequencing methodology.

Results: Diagnosis of AIHA was found in 10% of the newly diagnosed treatment naïve CLL patients observed at our institution. Seventy percent of the patients had unmutated immunoglobulin heavy-chain variable region gene (IGHV). The genetic results presented the most frequent unfavorable cytogenetics with 11q deletions and NOTHCH mutation. Time to treatment failure (TTF) was 11,3 months and Overall Survival (OS) of CLL/AIHA was 35,9months.

Conclusion: This study points out that AIHA is a rare event in CLL with a significantly higher incidence in patients with unmutated IGHV genes subgroup IGHV1-69 and adverse genetic profile with 11q deletions and a NOTHCH mutation. The results of our study are consistent with already published studies covering specific molecular signatures.

Keywords: CLL, AIHA, unmutated IGHV, del11q

Introduction

Chronic lymphocytic leukemia (CLL) as part of the group of lymphoproliferative disorders is characterized by presence of autoimmune phenomena attributable to underlying dysfunctions of the immune arrangement. CLL is commonly associated with three autoimmune disorders: autoimmune hemolytic anemia (AIHA), idiopathic thrombocytopenic purpura, and pure red cell aplasia [1,2].

From the large group of lymphoproliferative diseases, CLL is the most often associated with AIHA. Immune dysfunctions are responsible for the occurrence of AIHA in 3 to 37% of patients with CLL [3].

Data from previous studies have shown that AIHA is more common in CLL with advanced stage of the disease [3]. According to the National Cancer Institute (NCI)-Sponsored Working Group Guidelines for CLL precisely AIHA is one of the indications for treatment in patients with CLL. Certain therapeutic modalities such as radiotherapy or therapy with alkylating agents may [4] have been considered as risk factors for the occurrence of AIHA.

As therapy proceeds an imbalance between the lymphocyte subsets may occur, which may result in the appearance of an autoimmune clone. Although investigated in a series of studies the exact mechanism for the occurrence of the autoimmune mechanism is not yet recognized [5].

AIHA is more frequently found in patients with unfavorable biological risk factors for CLL with unmutated IGHV gene (polyreactive BCR which recognizes auto-antigens).

B lymphocytes at CLL are responsible of pathogenic mechanisms, involving aberrant antigen presentation and cytokine production. CLL cells may process red blood cell antigens and act as antigen presenting cells, inducing a T-cell response and the formation of polyclonal antibodies by normal B cells, thus indirectly provoking autoimmune hemolytic anemia. CLL cells express inhibitory cytokines that alter tolerance, which may facilitate the escape of self-reactive cells [6].

Efremov et al. [7] reported that in approximately half of AIHA/CLL patients there is a preferential expression of 2 IGVH gene segments, 51p1 and DP-50, in association with a particular CDR3 region by the leukemic cells, suggesting the possibility that CLL cells may be directly involved in the pathogenesis of AIHA. However, in the majority of AIHA cases the autoantibodies are of the IgG class. It is unlikely that such autoantibodies may represent the direct product of the CD5 B-cell clone. Since IgG autoantibodies are in fact almost always polyclonal.

Therapeutic approaches, such as alkylating agents, particularly purine analogues, have been considered as risk factors for the occurrence of AIHA. It is thought that the imbalance among lymphocyte subsets, contributed by therapy, could result in the emergence of an autoimmune clone. However, the exact mechanisms leading to autoimmunity in CLL are still unclear and have been the subject of several biologic studies.

Material and methods

This is a retrospective study of 100 patients with CLL, diagnosed and followed in the period between January 2012 and January 2022. Traditional laboratory, clinical and biological prognostic factors were evaluated at first patient visit to University Clinic of Hematology -Skopje Macedonia. The diagnosis of patients with CLL was set according to the recommendations of the International Working Group on CLL(IWCLL) [8].

Medical data of all the patients were implemented from the medical history of the disease. Physical examination with a two-dimensional diameter of the enlarged lymph nodes in all regions available for palpation (neck, axillar, supraclavicular, inguinal, femoral) was performed.

The dimensions of the spleen and the liver are noted by physical examination and ECHO of the abdominal organs, computerized tomography (CT) scan of chest and abdomen were used. Performance status was assessed by Eastern Cooperative Oncology Group (ECOG) Performance Status. A complete blood count of leukocytes, platelets, hemoglobin value and a differential blood count with percentage and absolute number of lymphocytes and reticulocytes were assessed. Biochemical markers with prognostic significance were investigated: serum level of bilirubin, LDH level, beta-2 microglobulin (B2M). Clinical stratification was carried out according to the BINET system.

Immunohematology analysis was performed at the Institute for Transfusion, Skopje, North Macedonia. Using a broad-spectrum antiserum the direct antiglobulin test (DAT) detected irregular antierythrocyte antibodies and the complement bound to the red cell membrane.

The presence of erythrocyte agglutination was considered a positive result. Irregular antierythrocyte antibodies in the serum were detected by the indirect antiglobulin test (IAT), the presence of erythrocyte agglutination was considered a positive result.

In all CLL diseased patients with evidence of anemia (hemoglobin< 12 g/dL) or one or more laboratory signs of hemolysis (increased bilirubin, increased lactic dehydrogenase, increased percent of reticulocyte) an immunohematology workup was performed. Patients showing anemia, associated with the presence of antierythrocyte autoantibody (AeAb), were considered as CLL with AIHA patients (CLL/AIHA).

A CLL diagnosis was assessed at the Immunohematology laboratory at the University Clinic for Hematology using ERIC panel and standard operating protocol (9) for flow cytometry. The BD FACSLyric™(BD-Biosciensis, San Jose, CA, USA) was analyzed using the panel as presented on table 1.

Table 1. CLL diagnosis panel of monoclonal antibodies for flow cytometry.

APC	**FITC**	**PE**	**PerCP**
Membrane markers			
CD45	CD5	CD19	
CD19	CD5	CD23	CD200
CD19	CD10	CD22	
CD19	CD20	CD3	
CD19	FMC7	CD79b	
CD19	CD38	CD138	
CD19	kappa	lambda	
CD19	CD43	CD81	
CD3	CD4	CD8	

Allophycocyanin (APC), Fluorescein isothiocyanate (FITC), phycoerythrin (PE), Peridinin-Chlorophyll-Protein (PerCP)

The histograms were interpreted using BD FACSuite Software. The positive expression of antibodies was considered as an expression of > 20% of mononuclear cells.

The Center for Bimolecular Pharmaceutical Analyses at the Faculty of pharmacy, Skopje, Republic of North Macedonia analyzed the individual data from 100 treatment naïve CLL patients, and the mutational status of the configuration of IGHV-IGHD-IGHJ rearrangements and genetics using reverse transcriptase – polymerase chain reaction (RT-PCR) and sequencing methodology.

The analyses were performed on mononuclear cells obtained from peripheral blood samples by Ficoll density gradient centrifugation. Total RNA was extracted using TRIzol reagent (Ambion, Life Techlogies) and reverse-transcribed using MuLV reverse transcriptase (Applied Biosystems, Foster City, CA, USA) and random hexamer primers, according to manufacturer's instructions.

IGHV-IGHD-IGHJ gene rearrangements were amplified by RT-PCR using a mixture of 5' primers specific for leader sequences of IGHV1 to IGHV6 subgroups 25 in conjunction with mixed 3' primers complementary to the germ line IGHJ genes. A 26 reverse transcriptase–polymerase chain reaction (RT-PCR) was carried out in a final volume of 25 μL with 10 pmol of each primer, 200 pmol of each deoxyribonucleotide, and 2.5 U Tag Gold Polymerase (Applied Biosystems, Foster City, CA, USA). Amplification consisted of an initial denaturation step of 10 minutes at 95°C, followed by 35 cycles of 95°C for 45 seconds, 60°C for 45 seconds, and 72°C for 1.5 minute, with a final extension step of 10 minutes.

Clonal PCR products were purified using low melt agarose and were sequenced with a reverse primer with BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). They were purified with BigDye X Terminator Purification Kit (Applied Biosystems, Foster City, CA, USA) and were run on 3500 Genetic Analyzer (Applied Biosystems).

Mutational status was calculated as a percentage of deviation from the closest germ line IGHV gene. Sequences with a germ line identity greater than or equal to 98% were considered unmutated and those with an identity less than 98% were considered mutated.

DEFINITION OF RESPONSE OF AIHA AND CLL

The AIHA response was assessed according to the weekly evaluation of the hemoglobin values combined with the monthly evaluation of the Coombs test.

The following criteria were applied to define the response of AIHA:

1. patients with no detectable AeAb and persistent hemoglobin values of 12 g/dL or higher were considered as complete responders (CR);
2. patients with persistent AeAb but with a hemoglobin increase to 12 g/dL or higher or of at least 3 g/dL were considered as partial responders (PR);
3. patients with persistent AeAb in the absence of a significant hemogloblin increase (< 3 g/dL) were considered as "failures."

In patients with a persisting response of AIHA (CR/PR), the immunohematologic follow-up evaluation was performed every 3 to 6 months [8].

SUPPORTIVE CARE

Considering the risk of opportunistic infections due to the underlying disease and to the therapy itself, all patients received trimethoprim-cotrimoxazole as prophylaxis against Pneumocystis carinii infection from the start of the steroid therapy.

Filtrated packed red cells were infused in the presence of severe and symptomatic anemia.

Results

An AIHA diagnosis was found in 10% of the newly diagnosed treatment naïve CLL patients. The median age was 66,4 years (range, 60-76 years), 90% of the CLL patients with AIHA were male. The median hemoglobin value at the time of AIHA diagnosis was 6,9 g/dL (range, 4-8 g/dL), other hematologic parameters are presented in Table 2.

Table 2. Hematologic parameters of CLL/AIHA patients

Hematologic parameters	**Range**	**Median value**
White blood cell	4-10 x10 ^{3}/uL	72,7 x10 ^{3}/uL
Absolute lymphocyte number	1,2-3,4 x10 ^{3}/uL	67,7x10 ^{3}/uL
Percentage of Reticulocytes	0,5-2,5%	8,6%
Platelets	150-450 x10 ^{3}/uL	130x10 ^{3}/uL

Distribution of patients according to BINET stage is presented in Figure 1. According to the BINET staging system 60% of the patients had BINET B stage.

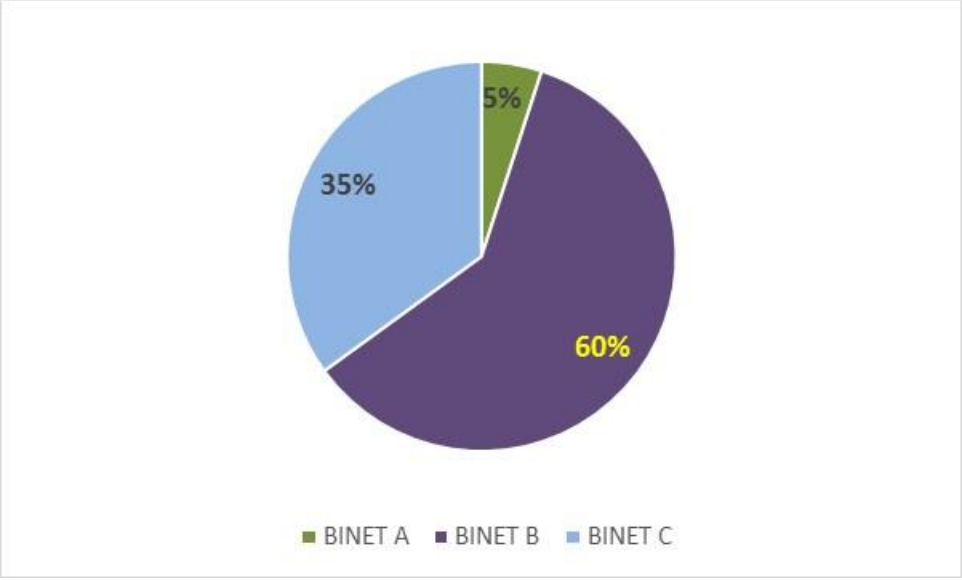
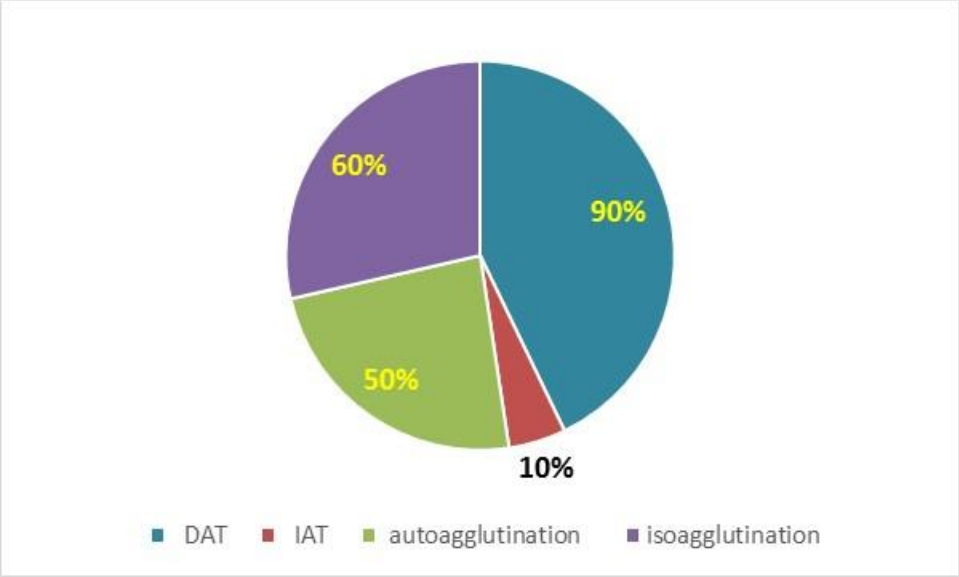


Figure 1. Distribution of patients according to the BINET stage.

Most of the patients (90%) had positive direct antiglobulin test (Coombs test), only 10% of patients had indirect antiglobulin test (Coombs test) positive, as immunohematology findings are presented in Figure 2.



DAT-direct antiglobulin test, IAT- indirect antiglobulin test

Figure 2. Distribution of patients according to the Coombs test.

All CLL/AIHA patients had CLL immunophenotype with triple positive expression of CD5, CD19, CD23 and 60% of patients had positive expression of marker with inferior survival CD38, distribution of patients with CD38 expression is presented in Figure3.

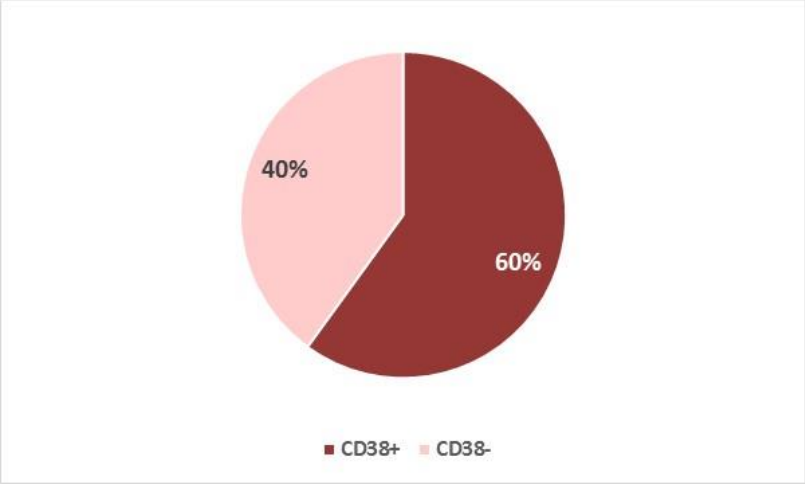
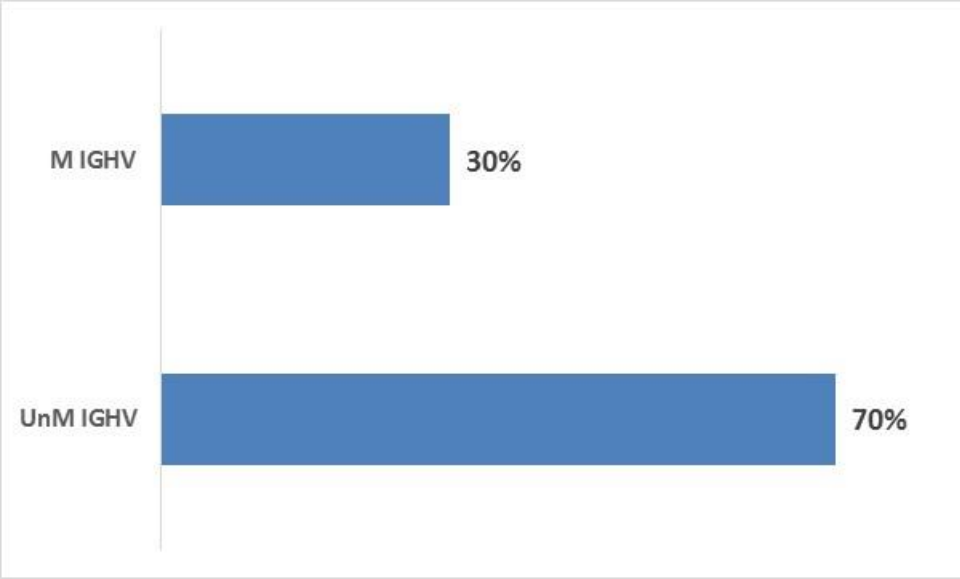


Figure 3. Distribution of patients according to CD38 expression.

The distribution of patients according to the mutational status of IGHV, which shows that 70% of patients with CLL/AIHA had unmutated IGHV, is presented in Figures 4-7. Most frequently expressed group at V gene was IGHV 1-69 gen, found at 71% of CLL/AIHA patients. The figure shows that 60% of patients had IGHD gene 3-3 and 65% had IGHJ-5*02 gen.



UnM IGHV- unmutated immunoglobulin heavy-chain variable region gene,
M IGHV- mutated immunoglobulin heavy-chain variable region gene

Figure 4. Distribution of patients according to mutational status of IGHV.

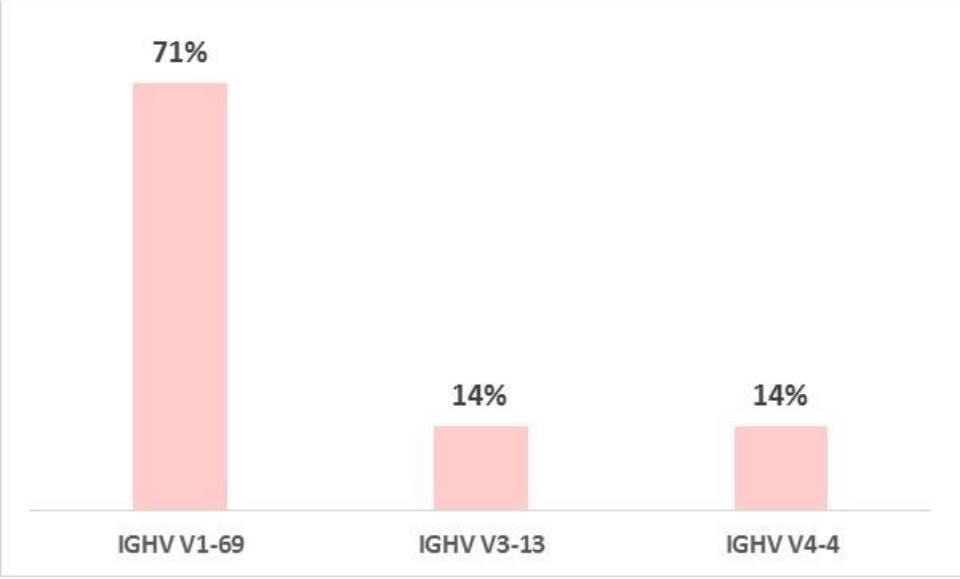


Figure 5. Distribution of patients according to V gene.

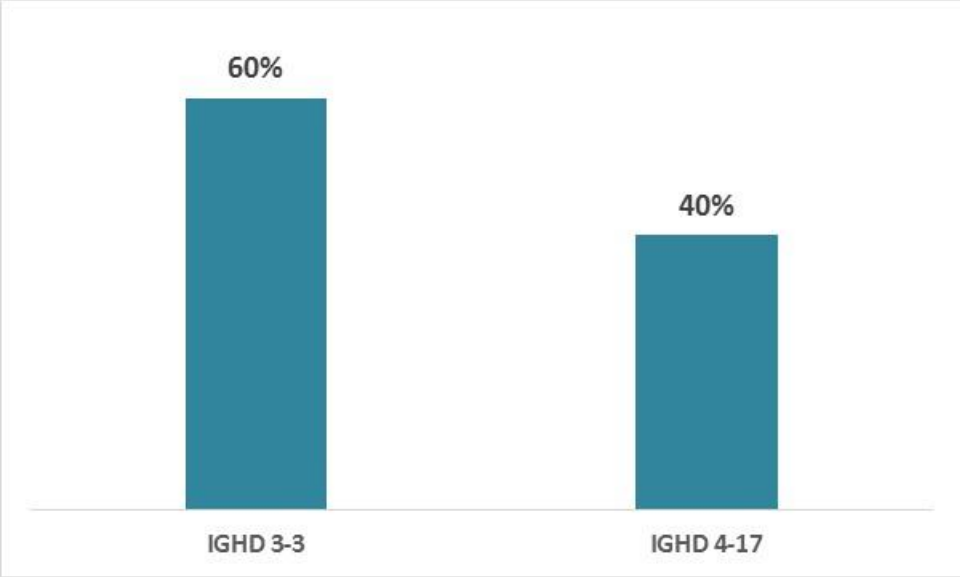


Figure 6. Distribution of patients according to D gene.

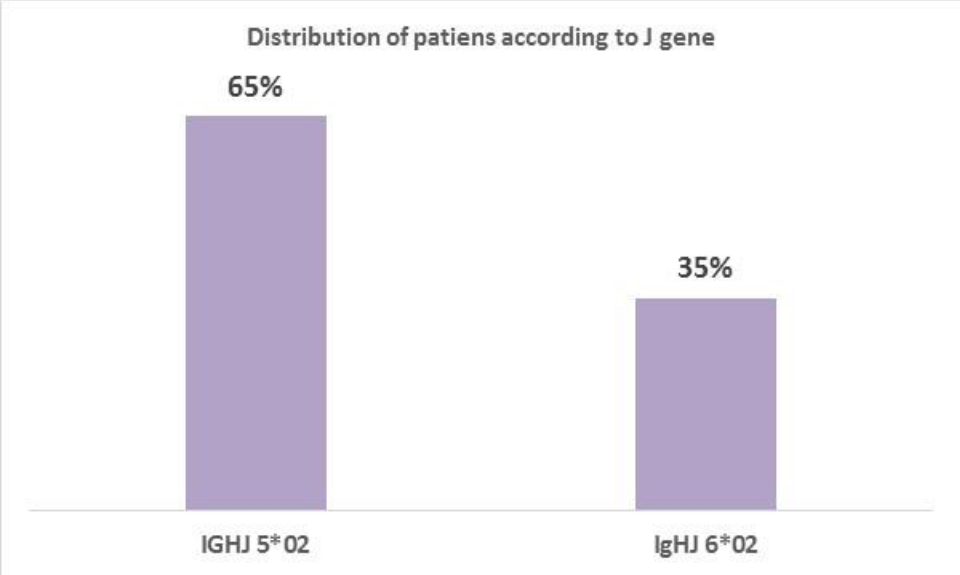


Figure 7. Distribution of patients according to J gene.

The genetic signature has presented unfavorable cytogenetics with 11q deletions found at 40% of CLL/AIHA patients and NOTHCH mutation at 30% of patients.

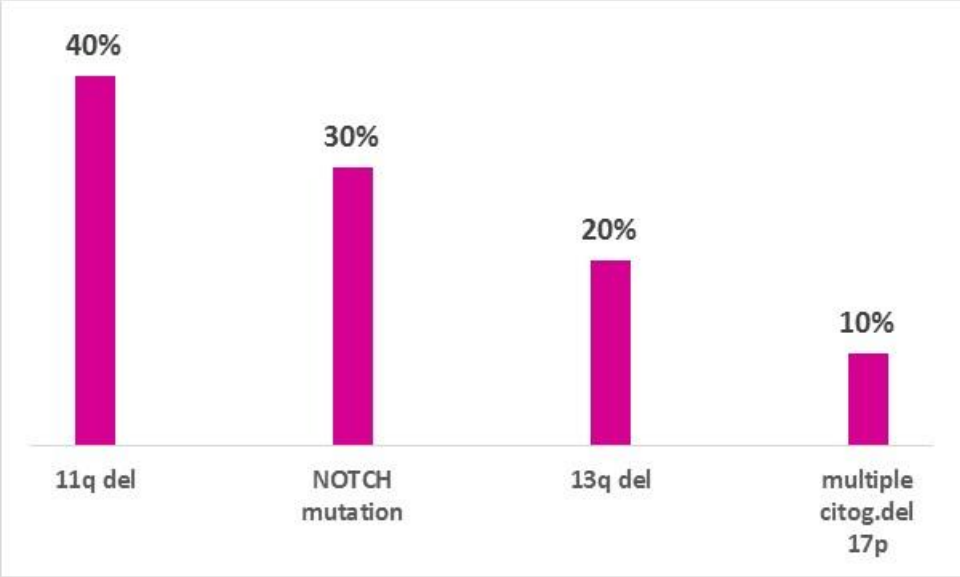
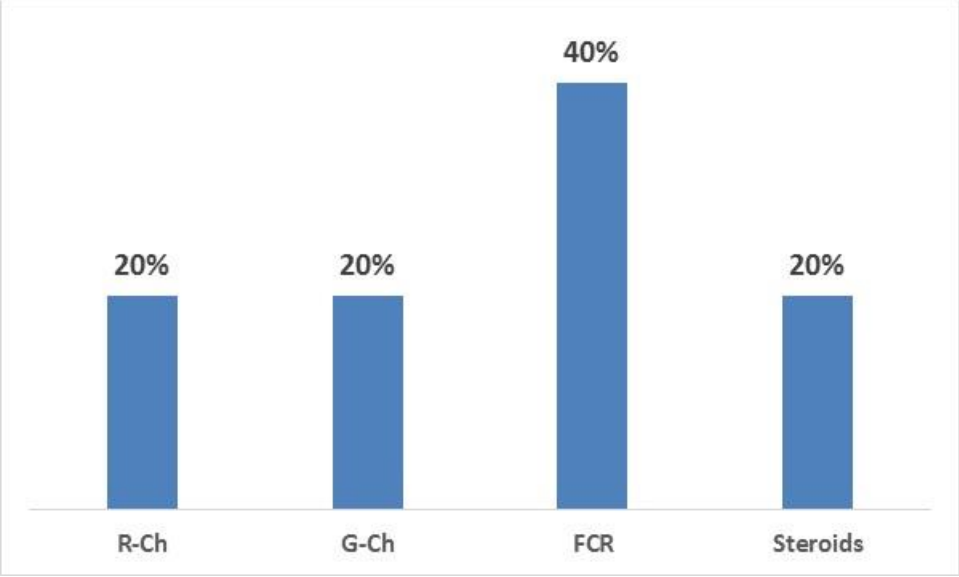


Figure 8. Distribution of patients according to genetic results.

The Protocol of Immunochemotherapy with Fludarabine, Cyclophosphamide with anti CD20 monoclonal antibody- Rituximab (FCR) was the most ordinated at 40% of CLL/AIHA patients, while 20% of patients were treated with steroids, Rituximab with Chlorambucil or anti CD20 monoclonal antibody- Gazyva (Obinutuzumab) with Chlorambucil (Figure 9).



R-Ch (Rituximab, Chlorambucil); G-Ch (Gazyva (Obinutuzumab), Chlorambucil), FCR (Fludarabine, Cyclophosphamide, Rituximab);Steroids(corticosteroids)

Figure 9. Distribution of patients according to therapy.

Figure 10 presents the Survival Distribution with Time to treatment failure (TTF) of 11,3 months, and Overall survival (OS) of 35,9 months.

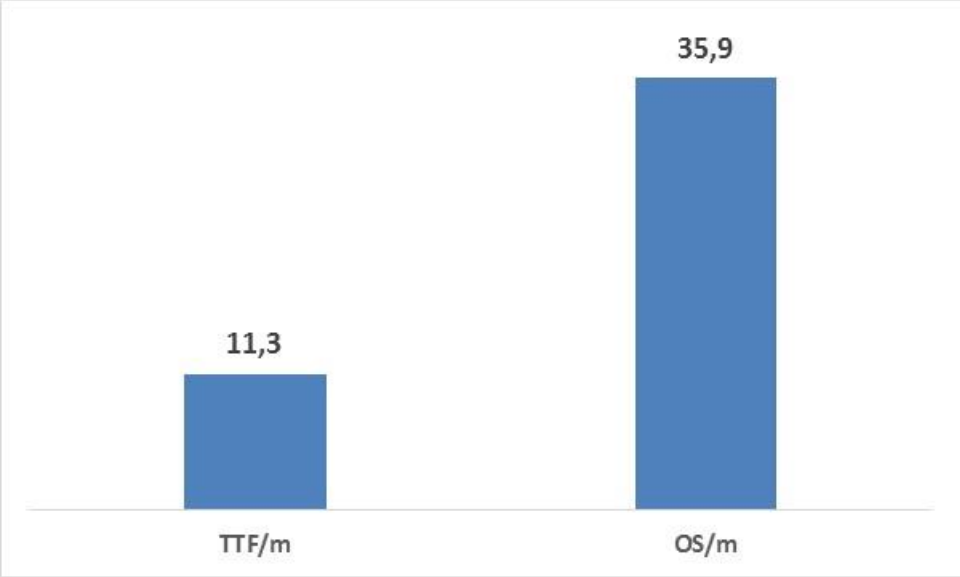


Figure 10. Survival distribution according to TTF and OS in months.

Most of CLL/AIHA patients (50%) had only one episode of autoimmune phenomena, while 40% of CLL/AIHA patients were alive at the conclusion of the study, and 10% of patients died with active autoimmune disease (Figure 11).

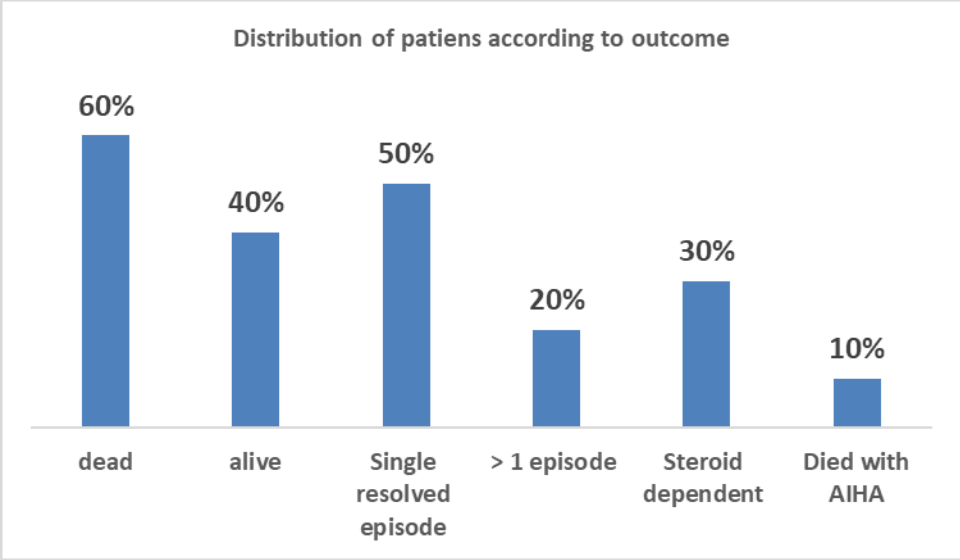


Figure 11. Distribution of patients according to survival status.

Discussion

We have described in a series of 100 CLL cases observed at a single institution. The overall rate of AIHA in our series of 100 CLL patients was 10%, and is higher then the previously reported by Hamblin

et al (5%) and De Rossi et al (6%)(10,3). The higher rates of AIHA in our serie probably reflect the prevalence of cases with an advanced stage of the disease, containing both the active and the stable disease component. Men, aged patients, and patients with a higher lymphocyte count showed a significantly higher rate of AIHA.

Our study presented the IgG class of the AeAb and the occurrence of AIHA at the time of CLL diagnosis with DAT in majority of patients.

Although we have no explanation for the higher rate of AIHA among men, the higher number of AIHA among elderly patients is not surprising. A decrease of the T-lymphocyte function and production may reflect the incapacity of the aged thymus to support an adequate generation and differentiation of T lymphocytes [11].

In our series, the large majority of patients showed clinical signs of active CLL at the time of AIHA diagnosis and, after therapy, achieved a response of both CLL and AIHA. This clinical finding indicate a very close relationship between the activity of CLL and AIHA. This may be explained by the biologic correlation between CLL and autoreactivity, because CD5+ B cells are involved in both [12].

Distribution of patients according to mutational status in our series of CLL patients with AIHA presented that most of the patients have adverse prognostic biological signature with unmutated IGHV. Most frequently expressed group at V gene was IGHV 1-69 gen, associated with IGHD gene 3-3 and J 5, 6 genes. The unmutated subset (U-CLL), of inferior prognosis, appears to derive from a pregerminal center B cell [13] which may also explain the occurrence of this autoimmune phenomenon. Patients with 11 q deletion, and NOTCH mutation were most affected by the occurrence of autoimmune hemolytic anemia.

After therapy, 50% of patients achieved the disappearance of the AeAb. However, this does not always correspond to the complete eradication of a specific B-lymphocyte clone producing the AeAb; 20% of responder patients, in fact, relapsed. It is of biologic interest that in 3 cases a different Ig class of autoantibody was detected at the time of AIHA relapse. This finding could suggest the presence of an immune dysregulation background in CLL patients developing AIHA, rather than a relapsing single autoimmune B-cell clone.

In our series of CLL/AIHA patients the time to treatment failure was shorter than a year because according to the recommendations this autoimmune phenomenon is an indication for starting therapy.
More extensive statistical analyses were not performed due to the small number of patients, and represents a negative component of our study.

Conclusion

This study point out that AIHA is a rare event in CLL with a significantly higher incidence in patients with unmutated IGHV genes subgroup IGHV1-69 and adverse genetic profile with 11q deletions and NOTHCH mutation. The results of our study are consistent with already published studies covering specific molecular signatures. AIHA in CLL patients affects their survival probability. Immunochemotherapy modalities with corticosteroids may be considered a potentially successful therapeutic approach for the management of patients with AIHA/CLL.

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