Journal of the Egyptian Society of Haematology & Research, Vol. 5, No. 1, March: 1-7, 2009

CLLU1 Gene Levels Quantitation as an Assessment Tool in Predicting the Time to Initiation of Therapy in Egyptian CLL Patients

DINA H. EL-DAHSHAN, M.D.*; RANIA M. GAWDAT, M.D.*; AMIRA A. HAMMAM, M.D.*; SHEREEN M. EL-HOSEINY, M.D.* and HASAN M. METWALY, M.D.**

The Departments of Clinical Pathology, Faculty of Medicine, Beni Suef University and Clinical Oncology, Faculty of Medicine, Minoufiya University***

ABSTRACT

Background:

Chronic lymphocytic leukemia (CLL) is an incurable disease with a highly variable clinical course. An estimation of survival or time to treatment in patients with CLL may be achieved based on numerous clinical, cell based, and molecular prognostic markers. A recent identified novel CLL-specific gene (CLL up-regulated gene 1, CLLU1), is exclusively up-regulated in CLL cells and its expression levels are important to evaluate the progression of the disease.

Aim of the Study:

To investigate CLLU1 expression in de novo Egyptian CLL patients and to evaluate its relation to the start time of treatment as a potential prognostic parameter.

Patients and Methods:

A cohort of 40 untreated CLL patients was studied. The expression levels of CLLU1 transcript c DNA was determined by quantitative real time-polymerase chain reaction. The relation between CLLU1 expression and time to initiation of therapy was analyzed.

Results:

There was high statistically significant difference between all patients when compared to controls as regards the expression level (p-value <0.001), with patient median expression showing 78.78 fold increase than the median controls levels. The median level of CLLU1 in cases who need treatment is more than those who did not take treatment with 36.75 fold increase and high statistically significant difference (p-value <0.001).

Conclusion:

In conclusion, our findings showed that the level of expression of CLLU1 gene may be used as an estimation of the time to initiation of therapy. Accordingly it may be used for prediction of the clinical course of the disease and hence prognosis.

Key Words: *CLLU1 gene – CLL – QRT-PCR.*

INTRODUCTION

Chronic lymphocytic leukemia (CLL) is one of the most common lymphoid malignancies accounting for approximately 11% of all hematologic neoplasms **[1]**. It is the most common leukemia in the western world with an annual incidence of 5/100000. The clinical course of the disease is heterogeneous, with some patients experiencing rapid disease progression and others living for decades without requiring treatment **[2]**.

The prognosis of patients with B-CLL has long been determined by the clinical staging systems of Binet and Rai **[3]**. Unfortunately, these systems are unable to identify patients in the early stages whose disease will rapidly progress. Neither can this approach clearly predict the response of individual patients to specific therapies **[4]**. A few other prognostic factors have been identified which are independent of clinical stage: Lymphocyte doubling time, pattern of bone marrow infiltration, LDH, serum B2 microglobulin, soluble CD23, serum thymidine kinase, expression of CD38 on CD 19 positive B-cells, genetic defects in specific molecular pathways, involving p53 at 17p13, or ATM at 11q22 and the mutational status of immunoglobulin heavy chain variable region genes (IgVh) **[5-9]**.

Clinical features and molecular/biologic factors such as ZAP-70, and cytogenetic abnormalities on fluorescent in situ hyperdization (FISH) have been found to be robust predictors

of treatment-free survival and overall survival among newly diagnosed patients **[10]**.

While cytogenetics and IgVh mutational status are good predictors of outcome, their determination is confined to specialized centers **[11,12]**. The identification of other markers which can be determined by monoclonal antibodies or by polymerase chain reaction would improve the diagnosis of CLL. Therefore, the major aim in the management algorithm of an indolent disease like CLL is to search for novel predictors of outcome and to develop non-toxic, target-specific therapies **[13]**.

One of the recent discovered genes is chronic lymphocyte leukemia up-regulated gene 1 (CLLU1) which was cloned and identified by Buhl et al., 2006 **[14]**. CLLU1 gene is located at chromosome 12q22, its transcripts have no significant similarity to other human genes and most of transcripts appear to be non-coding while no miRNAs were detected, nevertheless, the non-coding transcripts may have functions that presently have not been described.

The gene is not conserved in other species and even though several of the putative CLLU1 splice variants contain a putative open reading frame of 121 amino acids, researchers have not able to convincingly detect expression of the putative CLLU1 protein in CLL patient samples. Thus, they do not know whether CLLU1 plays a role in the pathogenesis of CLL, or whether its expression is a reflection of other events in the CLL cells **[15,16]**.

The goal of the current study is to detect the prevalence of CLLU1 gene expression among 40 de novo Egyptian CLL patients as a molecular marker that may be useful in the prediction of the time to initiation of therapy and useful in prognosis.

MATERIAL AND METHODS

Patients and methods:

Fourty Egyptian Patients with the diagnosis of Chronic lymphocytic leukemia (CLL) referred to the Kasr El-Aini Center of R adiation, Oncology and Nuclear Medicine (NEMROCK) and Beni Suef University Hospital between January 2007 and December 2008 were included in this study.

Peripheral blood (PB) and bone marrow samples from these patients were collected at diagnosis and centrifuged to obtain mononuclear cells (MNCS).

The diagnosis of CLL was made based on the standard morphologic and immunophenotypic criteria including absolute lymphocytosis, presence of basket cells and positivity for CD5+ and CD19+. Clinical data were obtained from the patients' medical records.

Ten age and sex matched individuals with lymphocytosis were included as controls.

CLLU1 gene was analyzed using real time quantitative reverse transcriptase polymerase chain reaction (RTQ-PCR) to investigate a possible relation, association, or correlation with the clinical features of patients at diagnosis (such as: Gender, age, hemoglobin (Hb), TLC, platelets count) and with treatment outcome and prognosis.

RNA isolation and real-time quantitative RT-PCR:

Mononuclear cells (MNCs) were isolated from 2ml peripheral blood at diagnosis by Ficoll density gradient centrifugation. Total RNA was extracted from MNCs using a QIAamp RNA blood kit (Qiagen,Germany). Complementary DNA (cDNA) was synthesized using (dt) 15-mer primer by superscript III Reverse transcriptase and stored at –20°C till use.

The mRNA expression levels of CLLU-1 gene and β2 microglobulin were measured by quantitative RT-PCR using an ABI PRISM 7000 Sequence Detector System (Applied Biosystems, Foster City, CA, USA). The quantitative RT-PCR amplification was performed using one of the CLLU1 gene transcripts named cDNA1 having the following primer and probes: ´5´-AGCTTGCAGATGGCAGATCA-´3´ (forward primer), ´5´-CATAAAGGGCAGCGAAATGC-´3´ (reverse primer) and ´5´-TATCTCCAGGCC-TTTCATTGGGTCAGGT- ´3´ (FAM-probe).

And for β2-microglobulin: ´5´-TGACTTT-GTCACAGCCCAAGATA-´3´ (forward primer), ´5´-AATCCAAATGCGGCATCTTC-´3´ (reverse primer) and ´5´-TGATGCTGCTTACATGTCTC-GATCCCA-´3´ (FAM-probe). All probes were TaqMan probes obtained from Applied Biosystems.

All reactions were perfomed in triplicate using 50ng c DNA and one-step RT-PCR Mix reagents (AB4309169) as described by the manufacturer. Primer and probe concentrations were 200 and 100nm, respectively. The reaction protocol used involved heating for 2 minutes at 50°C and 10 minutes at 95°C, followed by 40 cycles of amplification (15 seconds at 95°C and 1 minute at 60°C). Analysis was performed using ABI PRISM 7000 Sequence Detection Software (Applied Biosystems).

The expression levels of CLLU-1 gene in tested samples were expressed in the form of CT (cycle threshold) level then normalized copy number (relative quantitation) was calculated using the Δ Δ CT equation. A negative control without template was included in each experiment.

Expression level of CLLU-1 was correlated with the clinical features of the studied patients at diagnosis including: Age, gender, TLC, hemoglobin, platelets, etc.

Differences in expression levels of CLLU-1 with respect to the prognostic factors were analysed using the Mann-Whitney U test. The expression levels were represented as the mean or median values. Results were considered significant at $p<0.05$.

For univariate CLLU1 analysis, time to treatment was also considered an end-point, defined as time from diagnosis to first treatment or end of follow-up.

RESULTS

The clinical characteristics of the study cohort are presented in Table (1). The median age at diagnosis was 50 years, with a mean of 53.5±9.5 and a range of 44-65 years.

Of the fourty patients included in this study 19 (47.5%) were males, while 21 (52.5%) were females.

The mean total leucocytic count (TLC) of the patients at diagnosis was 57±53.6x10^9/L (range 3.4-110x10^9/L) with mean lymphocytic percentage 70.5±22.5% (range 45-90%), the mean hemoglobin 10.7±4gm/dl (range 6.3-14.8 gm/dl), mean platelet count 181.6±130x10^9/L (range 58-520x10^9/L).

Thirty of 40 patients (75%) had hepatosplenomegally (HSM), while 25/40 (62.5%) had lymphadenopathy.

According to Rai staging system which stated that stage 0=lymphocytosis with 40% of BM cells lymphocytes, stage I=stage 0 + enlarged LN, stage II=stage I + enlarged liver, spleen or both, stage III=stage II + Hb<11gm/dl and stage IV=stage III + platelets <100x10^9/L), 21 patients were in stage II, 9 patients were in stage III, and 10 patients were in stage IV.

CLLU1 gene was expressed in 38/40 (95%) patients at diagnosis with a median level of 333.1 and mean of 14179.74±14179.212 (range 0.5285-42642.37). While the median level was 4.2281, with mean level 5.86±5.8593 (range 0.0007-14.7230) in controls.

There was high statistically significant difference between all patients when compared to controls as regards the gene expression level ($p<0.001$) with patients median expression level showing 78.78 fold increase than median controls level.

The follow-up of patients from January 2007 to December 2008 showed that 18/40 cases showed disease progression and started treatment, while 22/40 cases did not show any change in disease activity and needed no treatment. The median CLLU1 level in cases who needed treatment was 8079.21, while the median CLLU1 level of those who did not need treatment was 219.79 with 36.75 fold increase in the treated cases than the untreated cases and with high statistical significant difference ($p< 0.001$).

When correlating the relative quantitation (RQ) level of CLLU1 gene with the lag period before starting treatment (weeks) there was negative correlation ($r=–0.557$) but high statistical significant difference ($p=0.016$).

There was no statistically significant difference when comparing patients (treated or untreated) with different clinical and laboratory findings including age, gender, total leucocytic count, hemoglobin level, platelet count, lymphocytic count, hepatosplenomegally or lymphadenopathy ($p>0.05$).

Table (1): Characteristics of 40 CLL patients.

Characteristic	Value
Gender, no (%)	
Male	19 (47.5%)
Female	21 (52.5%)
Age at diagnosis (years)	53.5±9.5 years (44-65 years)*
Median	50 years
Total leucocytic count x10^9/L	57±53.6x10^9/L (range 3.4-110x10^9/L)
Hemoglobin gm/dl	10.7±4gm/dl (range 6.3-14.8gm/dl)
Plateletsx10^9/L	181.6±130x10^9/L (range 58-520x10^9/L).
Lymphocytic count (%)	70.8% (45-90%)
Absolute Lymphocytic count (Mean ± SD)	13868.3±7.5
Scoring of CLL no (%)	
II	21 (52.5%)
III	9 (17.5%)
IV	10 (25%)
Follow-up	
Needed treatment	18 (45%)
Did not need treatment	22 (55%)

* Mean ± SD (range).

Table (2): CLLU1 gene in 40 CLL patients compared to controls.

CLL U1 gene	Positive cases	Level of CLLU1 gene (median)	Fold increase	*p*-Value
Cases	38/40 (95%)	333.1	78.78	*p*<0.001
Controls	8/10 (80%)	4.2281		
Treated Cases	18/40 (45%)	8079.21	36.75	*p*<0.001
Untreated Cases	22/40 (55%)	219.79		

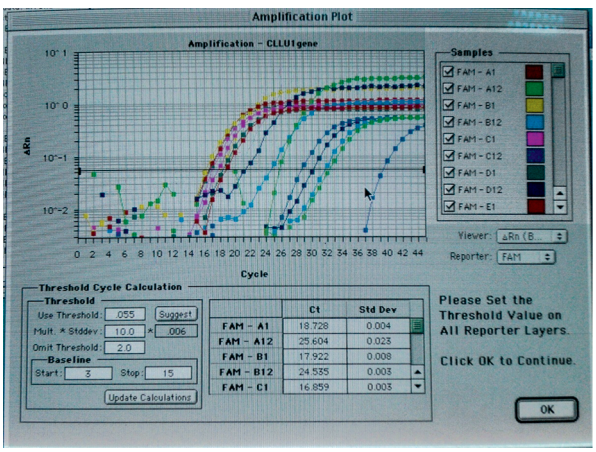

Fig. (1): CLLU1 gene in CLL cases and controls.

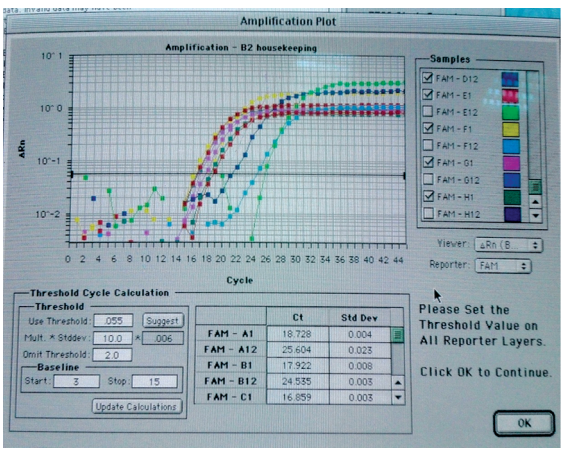

Fig. (2): Housekeeping gene (β2 microglobulin) in CLL cases and controls.

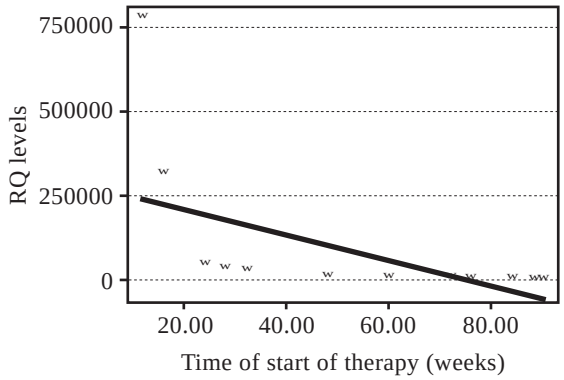

Fig. (3): Correlation curve between relative quantitation (RQ) levels of CLLU1 gene and time of start of therapy.

DISCUSSION

The origin of new genes is extremely important to evolutionary innovation. Most new genes arise from existing genes through duplication or recombination. The origin of genes from non-coding DNA is extremely rare, and very few eukaryotic examples are known in yeast and drosophilia **[16]**.

In this study, CLLU1 gene expression was analyzed in peripheral blood of de novo 40 CLL Egyptian patients in comparison to peripheral blood of 10 healthy controls, by using QRT-PCR. While many of the patients were presenting at the beginning with low grade CLL and are preferentially treated with a [watchful waiting] approach **[17]**, the patients were followed-up for two years, to observe who was in need for early treatment and compare the levels of CLLU1 in the patients who needed early aggressive treatment to those who stayed with stable disease.

Our results are in accordance with the earlier study **[14]**, which showed that the restricted high levels of CLLU1 was found in CLL when compared to normal B lymphocytes and other hematological disorders, also, this was confirmed by a recent study **[18]**.

Another study **[19]** reported a significantly high levels of CLLU1 gene in CLL patients using two QRT-PCR reactions, for c DNA and CDS (the most common splice variants of CLLU1 gene), where they found the median c DNA expression levels was 27.27 fold above the level found in normal B cells of healthy controls and a significant linear relationship between the expression level of c DNA and CDS within the patients was found. However, they recommend to use cDNA variant as a measure for the CLLU1 level which we used in our study, since the c DNA QRT-PCR reaction spans an exon-intron boundary and is thus not affected by contaminating DNA. No correlation was found between the patient's age of diagnosis and CLLU1 gene level, this was in line with our results.

In another work, CLLU1 expression levels represented a continuum ranging from 0.0005- to 10.000-fold up-regulation compared with that of normal B cells with a median of 22.9-fold up-regulation **[20]**.

In fact, the absence of expression in the other tissues raised the possibility that CLLU1 may not be required for normal human function and development and the reason for the high expression in CLL could be the result of accidental activation of a promotor upstream of CLLU1 **[19]**.

The specificity and the sensitivity of the CLLU1 expression as well as its utility in distinguishing B-CLL from other B-cell lymphoproliferative disorders was assessed **[21]**. CLLU1 expression levels were measured by QRT-PCR and the results showed over expression of CLLU1 in more than 85% of the cases of B-CLL in comparison to the healthy controls and other B-lymphoproliferative disorders **[21]**. CLLU1 expression was not detected in the majority of the lymphoma patients and these results were confirmed by Kienle et al. **[22]**, whose results suggested that analysis of CLLU1 expression by RT-PCR is a simple novel diagnostic tool especially in cases where known diagnostic parameters fail to establish the diagnosis.

In our study, there is association between the expression levels of CLLU1 and time to initiation of therapy during the study period which was found to be significantly higher in patients who received treatment early than those who did not need treatment (p-value<0.001).

Buhl et al. **[19]**, revealed that a doubling of c DNA transcript level was associated with an increased risk of initiation of therapy and this association may help to identify how soon a given CLL patient will be in need for therapy.

Low to moderate (≤40 fold increase than controls) CLLU1 expression is associated with an indolent disease that may never require treatment, whereas high (>40 fold increase than controls) CLLU1 expression is associated with a more aggressive clinical course with early therapy onset. Moreover its high expression has strong independent prognostic significance for overall survival in CLL.

The value of CLLU1 level estimation was that it was the first demonstration within the field of dose-response relationship between the expression level of a prognostic gene and time to therapy initiation **[19]**.

A continuous proportional relationship between the expression level of CLLU1 at time of diagnosis and the relative risk of early death was demonstrated **[20]**. Therefore, CLLU1 is a prognostic marker in CLL, as high expression levels are associated with shorter time to treatment and poor overall survival in patients **[19,20,23]**.

In a Chinese study, Chen et al. **[24]**, examined CDS transcript expression instead of c DNA1, CDS transcription was expressed in 50% of patients with CLL where it was negatively expressed in normal B-subpopulation and other hematological disorders as acute leukemia, multiple myeloma and polycythemia vera. They correlated CLLU1 expression to the prognostic factors of CLL as CD 38, IgVH somatic hypermutational status in CLL, ZAP-70 and cytogenetic aberrations with a conclusion that patient stratification according to the prognostic markers demonstrates a significant increase of CLLU1 expression in high risk groups and that the unique exclusive expression of CLLU1 might be an important prognostic factor in CLL.

Hayette and colleagues **[25]** confirmed the prognostic significance of CLLU1 in CLL and its usefulness as a reliable alternative to IgVh gene sequencing. Moreover, its quantification does not require purification of B lymphocyte, a significant advantage in routine practice.

The expression of CLLLU1 might play an important critical role in monitoring minimal residual disease and therefore might be used for follow-up of patients with CLL. In addition evaluation of CLLU1 levels in the blood is highly specific for detecting residual CLL with sensitivity that might preclude the need for marrow assessment of all patients **[26]**.

Buhl et al. **[14]** hypothesis is that if CLLU1 turns out to be involved in CLL pathogenesis, targeting of CLLU1, for example using siRNA **[27,28]** could represent an ideal strategy for development of CLL-specific therapy because such therapy would not affect other tissues and if CLLU1 does not have an important role in CLL development or progression, it may still be useful for targeting cell-suicide gene therapy to CLL cells **[29,30]**.

Buhl et al. **[15]** continued his studies to further investigate the biological properties of the CLLU1 m RNAs; their results suggested that all the splice variants are derived from one primary transcript and regulated by a common promotor upstream from Exon 1 and demonstrated that CLLU1 levels in serial CLL samples of untreated patients were stable over time and with similar levels in peripheral blood and bone marrow. Moreover, the CLL cells of anyone patient have uniform expression as assessed by in situ hybridization. The expression levels are similar in patient samples before treatment and after relapse; hence a high CLLU1 level did not appear to be a feature that is acquired by CLL clone during the course of the disease **[15]**.

The current standard of care of CLL is to initiate treatment when a patient has progressive or symptomatic disease **[31]**.

As of today, only two molecular CLL clone-specific features are known: The IgVh structure and CLLU1 expression level. These two parameters may be considered the fingerprint of a particular CLL clone **[15]**.

In conclusion, our finding further emphasized that the expression level of CLLU1 in newly diagnosed CLL patients give an idea about the time of initiation of therapy, accordingly it could be highly predictive of the prognosis and the clinical course of the disease. Further studies and more clinical trials should help elucidate the important function of this gene and elucidate the potential biological implications of detecting CLLU1 expression levels in CLL patients and its potential use as a therapeutic target.

REFERENCES

1- Jemal A, Seigel R, Ward E, et al. Cancer statistics, 2008. CA Cancer J Clin. 2008; 58: 71-96.

2- Dighiero C, Maloum K, Desablens E, et al. Cholorambucil in indolent chronic lymphocytic leukemia. French

Cooperative Groop On Chronic Lymphocytic Leukemia. N Engl J Med. 1998; 338: 1506-1514.

3- Kipps TJ. Chronic lymphocytic leukemia. Curr Opin Hematol. 2000; 7: 223-234.

4- Hauswirth AW, Jagar U. Impact of cytogenetic and molecular prognostic markers on the clinical management of chronic lymphocytic leukemia. Hematologica. 2008, 93 (1): 14-19.

5- Montserrat E, Sachez-Bsono J, Vinolas N, et al. Lymphocyte doubling time in chronic lymphocytic leukemia; analysis of its prognostic significance. Br J Hematol. 1986, 62: 567-575.

6- Reinisch W, Willheim M, Hilgarth M, et al. Soluble CD23 reliably disease activity in B-cell chronic lymphocytic leukemia. J Clin Oncol. 1994, 12: 2146-2152.

7- Hallek M, Langenmayer I, Nerl C, et al. Elevated serum thymidine kinase levels identify a subgroup at high risk of disease progression in early, nonsmoldering chronic lymphocytic leukemia. Blood. 1999; 93: 1732-1737.

8- Hamblin TJ, Orchard JA, Ibbotoson RE, et al. CD38 expression and immunoglobulin variable region mutations are independent prognostic variables in chronic lymphocytic leukemia, but CD38 expression may vary during the course of the disease. Blood. 2002; 99: 1023-1029.

9- Dohner H, Stilgenbauer S, Benner A, et al. Genomic aberrations and survival in chronic lymphocytic leukemia. N Eng J Med. 2000; 343: 1910-1916.

10- Shanafelt TD. Predicting clinical outcome in CLL: How and why. Hematology. 2009; 421-429.

11- Oscier DG, Gardiner AC, Mould SJ, et al. Multivariate analysis of prognostic factors in CLL. Clinical stage, IGVH gene mutational status, and loss or mutation of the P53 gene are independent prognostic factors. Blood. 2002; 100: 1177-1184.

12- Krober A, Seiler T, Benner A, et al. VH mutation status, CD38 expression level, genomic aberrations, and survival in chronic lymphocytic leukemia. Blood. 2002; 100: 1410-1416.

13- Gaiger A, Heintel D, Jager U. Novel molecular diagnostic and therapeutic target in chronic lymphocytic leukemia. European Journal of clinical investigation. 2004; 34 (suppl. 2): 25-30.

14- Buhl AM, Jurlander J, Jorgensen FS, et al. Identification of a gene on chromosome 12q 22 uniquely overexpressed in chronic lymphocytic leukemia. Blood. 2006; 107: 2904-2911.

15- Buhl AM, Novotny GW, Josefsson P, et al. The CLLU1 expression level is a stable and inherent feature of the chronic lymphocytic leukemia clone. Leukemia. 2009; 23: 1182-1186.

16- Knowles DG, McLysaght A. Recent de novo origin of human protein-coding genes. Genome research. 2009; 19: 1752-1759.

17- Van Bockstaele F, Verhasselt B, Philippe J. Prognostic markers in chronic lymphocytic leukemia: A comprehensive review. Blood Rev. 2009; 23: 25-47.

18- Roumelioti M, Palaiologou D, Strvodimos G, et al. Over expression of CLLU1 Gene: A unique characteristic of unmutated CLL lymphocytes. Haematologica. 2009; 93 (s3): S35.

19- Buhl AM, Jurlander J, Geisler CH et al. CLLU1 expression levels predict time to initiation of therapy and overall survival in chronic lymphocytic leukemia. Eur J Hematol. 2006; 76: 455-464.

20- Josefsson P, Geisler CH, Leffers H, et al. CLLU1 expression analysis adds prognostic information in chronic lymphocytic leukemia. Blood. 2007; 109 (11): 4973-4979.

21- Oppliger Leibundgut E, Dissler D, De Beer D, et al. CLLU1expression distinguishes B-cell lymphoproliferative disorders. Haematologica. 2007; 92 (Suppl 1): 0522.

22- Kienle D, Benner A, Laufle D, et al. Quantitative gene expression analyses of surrogate markers for genetic risk groups and survival in CLL. Haematologica. 2008; 93 (s1); 215: 0532.

23- Kaderi MA, Kanduri M, Mansouri M, et al. LPL is the strongest prognostic factor in a comparative study of RNA-based markers in chronic lymphocytic leukemia. Blood. (ASH Annual Meeting Abstracts). 2009; 114: 1254.

24- Chen L, Lianyong J, Zheng W, et al. The prognostic evaluation of CLLU1 expression levels in 50 Chinese patients with chronic lymphocytic leukemia. Leukemia and Lymphoma. 2007; 48 (9): 1785-1792.

25- Hayette S, Bastard C, Mozziconacci MJ, et al. Expression levels of CLLU1 and LPL new disease assessment tools in CLL patients in Binet stage A. Haematologica.

26- James D, Avery ED, Rassenti LA, et al. 3158 CLLU1, a novel molecular marker for minimal residual disease monitoring in chronic lymphocytic leukemia. 50th ASH annual meeting and exposition. 2008. Online program and abstracts.

27- Woessmann W, Damm-Welk C, Fuchs U, et al. RNA interference; new mechanisms for targeted treatment. Rev Clin Exp Hematol. 2003; 7: 270-291.

28- Mello CC, Conte DJ. Revealing the world of RNA interference. Nature. 2004, 431: 338-342.

29- Vasaux G, Martin-Duque P. Use of suicide genes for cancer gene therapy: Study of the different approaches. Expert Opin Biol Ther. 2004; 4: 519-530.

30- Wendtner CM, Kofler DM, Mayr C, et al. The potential of gene transfer into primary-CLL cells using recombinant virus vectors. Leuk Lymphoma. 2004; 45: 697-904.

31- Hallek M, Cheson BD, Catovsky D, et al. Guidelines for the diagnosis and treatment of chronic lymphocytic leukemia a report from the international workshop on chronic lymphocytic leukemia updating the National Cancer Institute Working Group 1996 guidelines. Blood. 2008; 111 (12): 5446-5456.