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Original article

A 57-gene expression signature in B-cell chronic lymphocytic leukemia

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Abstract

B-CLL is the most frequent type of leukemia in the Western countries. The disease, common among the elderly, follows a variable course in terms of survival time and symptoms. There is evidence that the accumulation of lymphocytes in peripheral blood and bone marrow is due to a cell resistance to apoptosis rather than to highly proliferative cells. Genetic mechanisms that lead to the development and progression of disease are mainly unknown, although a number of prognostically and diagnostically important genetic markers have been identified.

The aim of this study is to investigate the gene expression profile, by a specific chip for microarray analysis, in B-CLL lymphocytes with regard to factors involved in apoptosis cascade, signal transduction, purine metabolism enzymes, interleukin expression, enzymes involved in the responses to oxidative stress. We found relevant results in a set of 19 of the 57 genes considered. IMP dehydrogenase, adenine phosphoribosyltransferase, adenylosuccinate lyase, adenylate kinase, ADORA1, G-protein-coupled receptor kinase 6, Bcl-2-like 1 *isoform 2*, caspase 6, and 8 were found underexpressed; while ADORA3, Gars-Airs-Gart, adenylate kinase 3, adenylate deaminase, NMN adenylyltransferase, CD26, CD38, interleukins 18 and 4 were found overexpressed.

The microarray technique is a powerful method for identification of potential important diagnostic and prognostic markers, besides giving prominence to genes candidate for further studies.

Keywords: B-CLL; Microarray; Apoptosis

1. Introduction

The sequencing of the human genome with the development of highly advanced technologies has provided the tools for global analysis of gene expression. This development has widened and deepened the knowledge of mechanisms and pathway involved in cell life. Understanding gene expression is the key to understanding disease, in such a view it is important to know when and how a gene is expressed and how its expression is related to a metabolic pathway. For this purpose, new technologies have been developed along with bioinformatics resources, in order to study gene expression under controlled and unbiased conditions.

Microarray technology represents one of the most innovative tools to study gene expression, providing a lot of information and data points that need to be analyzed and interpreted by specific informatic tools. Such a technique can be employed in the investigation of pathologic alterations of cell function: in particular, many studies have stated the importance of its application in the analysis of gene expression profile in cancer cell with respect to the healthy counterpart [1]. The information obtained with this approach can lead to identification of diagnostic and prognostic markers, to a clinical staging and new therapies.

B-cell chronic lymphocytic leukemia (B-CLL) is the most common form of leukemia in elderly in Western countries, with a peak of incidence over 60 years; it is reported to be twice as common in men as in women. The disease is characterized by an accumulation of small, phenotypically mature, malignant lymphocytes in peripheral blood and bone marrow. This accumulation is not due to highly proliferative cells, but

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rather to a significant resistance to apoptosis [2,3]. Malignant B lymphocytes express a specific pattern of surface antigens: CD5, CD19, CD20, CD23, low levels of surface immunoglobulin (Ig), with κ or λ monoclonal restriction [4]. Clinical course of B-CLL is extremely variable; the heterogeneity in clinical behaviour characterizes prognosis and responsiveness to therapies. The underlying cause of B-CLL is still unknown; for this reason many studies have been focused to the identification of new clinical parameters for outcome prediction and disease classification, able to distinguish subsets with different prognosis. Some biological features have been identified as prognostic markers, such as lymphocyte doubling time, serum thymidine kinase level, CD38 expression levels and mutational status of the expressed immunoglobulin heavy-chain variable-region (IgV_H) genes: all these parameters have been coupled to the Rai and Binet staging systems. CD38 expression and mutation of IgV_H genes are significantly related with progression of disease and median survival. $CD38^+$ patients show unfavourable clinical course, characterized by advanced disease stage, poor responses to chemotherapy and therefore reduction of median survival [5–7].

ZAP-70 (*zeta-associated protein of 70 kDa*) is a potential marker in progress [8]: its expression is strongly associated with mutational status of the IgV_H genes, with a prognostic significance comparable to IgV_H mutational status [9,10]. ZAP-70 positivity-expression appears to identify a subset of patients with a more progressive form of disease. Moreover, it was demonstrated that ZAP-70/positive cells are more responsive to signals derived from their surrounding environment and have an increased propensity to respond to survival signals [11].

Although these considerations, the understanding of mechanisms involved in pathogenesis of the disease is far to be completed and the information acquired until today lead to suppose that CLL is a multifactorial disease caused by complex interactions among different pathways and factors. The aim of the present study is the analysis of gene expression profile in patients affected by B-cell chronic lymphocytic leukemia, with specific regard to cell life critical aspects, as apoptotic process, transduction signalling, interleukin expression, enzymes involved in purine metabolism and oxidative stress.

2. Materials and methods

2.1. Patients and study design

Gene expression analysis was performed on 5 B-CLL patients, attending a check-up every month at the Hematology Division of Siena University Polyclinic. The criteria of exclusion were: recent cytostatic treatment (less than 4 weeks at time of testing), a percentage of T cells $> 25\%$, an absolute value of lymphocytes <5000 and B-CLL patients who did not express CD5 surface antigen. Diagnosis was made by clinical and immunological criteria. Healthy blood donors ($n = 6$) were picked at random and served as source of normal peripheral blood B cells. Informed consent was obtained from all subjects. In the microarray analysis each patient was spotted versus the pooled healthy subjects.

2.2. Lymphocyte preparation

Total peripheral blood lymphocytes (PBL) from B-CLL patients were separated from heparinized venous blood by Histopaque 1077 (Sigma–Aldrich, St Louis, MO, USA) density gradient centrifugation. The normal control samples were received as enriched buffy coats; after PBL separation, $CD19^+$ B cells were further negatively purified, performing a depletion of CD2 positive cells with Dynabeads Pan M-450 CD2 (Dynal Biotech ASA, Oslo, Norway), according to manufacturer's indications. Following bead selection, the purity of $CD19^+$ cells typically exceeded 95%. B-CLL samples used throughout the study were not further manipulated after Histopaque purification, as the mean $CD19^+$ cell content was >94%.

2.3. RNA extraction, labelled cDNA preparation, hybridization

Total RNA was isolated from cells using QIAamp RNA Blood Mini Kit (Qiagen Inc., Valencia CA, USA) and suspended in RNAse free water in a final concentration of 1 μg/μl. RNA was quantified by reading the absorbance at A_{260}/A_{280} and the RNA quality was further tested using Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA).

The microarray studies were performed within the Isogen Biosolutions Hybridization Service (Ebersberg, Germany).

2.3.1. Microarray chip

According to our indications, a specific chip was prepared, with 57 genes coding for enzymes of the purine de novo, salvage and catabolic pathways, oxidative stress enzymes, signal transduction and apoptosis-related proteins. Specific oligonucleotides for each gene were chosen from GenBank and spotted in duplicate. *HGPRT*, *Phospholipase A2* and *Ubiquitin* were present as positive controls.

Starting from about 5 μg total RNA per sample, RT-PCR and labelling steps were performed using the ExpressArt mRNA Amplification Kit, Micro Version (AmpTec GmbH, Hamburg, Germany) and ExpressArt Aminoallyl mRNA Amplification Kit (AmpTec GmbH, Hamburg, Germany), respectively, according to the manufacturer manual. The dyes used for coupling were Cy3 and Cy5 Post-Labelling reactive Dye Packs (Amersham, Piscataway, NJ, USA). *Cy3*, which produces a green fluorescence, and *Cy5*, which produces a red fluorescence, were used for labelling B-CLL patient and healthy subject samples, respectively.

The hybridization was done using Ocimum Biosolutions' salt-based hybridization buffer on a Tecan HS4800 automated hybridization station. Hybridization was performed at 50 °C for 90 min, followed by washing with three different washing buffers (2× SSC 0.1% SDS, 1× SSC and 0.5× SSC) 5 min each.