Pathol. Oncol. Res. (2010) 16:213–216
DOI 10.1007/s12253-009-9209-2

IFN Gamma Gene Polymorphism May Contribute to the Susceptibility to CLL

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Received: 6 July 2009 / Accepted: 2 September 2009 / Published online: 15 September 2009

Abstract The pathogenesis of B-cell chronic lymphocytic leukemia (B-CLL) has been linked with the production and activity of certain growth factors. However a significant proportion of CLL patients display immune abnormalities suggestive of aberrant cytokine secretion and/or response. In contrast to B lymphocytes, T cells of B-CLL patients characterise with the increased production of interferon-gamma (IFN-γ) and this cytokine has been indicated to prevent malignant cells from entering apoptosis including the slowly expanding population of CD5+ B cells that characterizes chronic lymphocytic leukemia. The aim of the present study was to assess whether functionally relevant interferon-gamma gene (*IFNG*) polymorphism (+847 A/T) contributes to the pathogenesis of B-CLL. In total 110 individuals was investigates, including 61 CLL patients and 50 healthy individuals. The presence of the IFNG AA genotype was found to be associated with susceptibility to CLL (23/61 vs. 7/50, $p<0.005$, for patients and controls, respectively). This results suggest that individuals rather prone to the lower level of IFN-γ production (associated with the presence of the A allele) appear to be more susceptible to this malignant disease.

Keywords Chronic lymphocytic leukemia · Gene polymorphism · INF gamma

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Introduction

Chronic lymphocytic leukemia (CLL) is the most common type of leukemia in adults, accounting for up to 25% of all newly diagnosed leukemia. It is a systemic haematological malignancy that originates from B cells (B-CLL) [1]. Although B-CLL is classified as a non-Hodgkin's lymphoma, several issues make this leukaemia a unique entity among malignant lymphoma. The pathogenesis of B-cell chronic lymphocytic leukemia (B-CLL) has been linked with the production and activity of certain growth factors. However a significant proportion of CLL patients display immune abnormalities suggestive of aberrant cytokine secretion and/or response. Further, despite the long survival of CLL B cells in vivo, they die rapidly by apoptosis when placed into culture [1, 2]. This strongly suggests that factors, such as cytokines, present in the local milieu may be playing a protective role in vivo.

Changes in the cytokine network may be responsible for malignant cell accumulation in B-cell chronic lymphocytic leukaemia (B-CLL). Recent evidence indicates that the slowly expanding population of CD5+ B cells that characterizes chronic lymphocytic leukemia results primarily from defects in responses to cytokines that regulate apoptosis IL-4, TGF-beta, IFN-alpha, IFN-gamma [2].

Polymorphisms in the regulatory and intronic regions of several cytokines have been associated with differential

cytokine production. Among different cytokines of question IFN-gamma (IFN-γ) is indicated to prevent malignant cells from entering apoptosis. IFN-γ is secreted by subsets of activated CD4+ and CD8+ T cells as well as activated natural killer (NK) cells and exerts its enormous variety of effects via a specific IFN-gamma receptor (IFN-γR) [3].

Six allelic forms have been identified according to the presence of a variable number of CA repeats in the first intron, where another polymorphism has been detected at position +874 caused by a single base mutation (TT, TA and AA). A significant association between increased IFN-γ production and the presence of +874T has been demonstrated [3, 4]. There is an absolute correlation between the presence of T allele and the presence of the high-producing microsatellite allele [2]. This T to A polymorphism coincides with a putative NF-kappa B binding site which might have functional consequences for the transcription of the human IFN-gamma (IFNG) gene [4, 5]. Therefore both, the T to A polymorphism as well as the CA microsatellite markers could directly influence the level of IFN-gamma production.

Many cases of CLL have a non-aggressive course and often do not require treatment, while other cases exhibit rapid progression within several years. The standard clinical procedures to estimate CLL prognosis are the clinical staging systems developed by Rai and Binet. These systems define a) early stage (Rai 0, Binet A), b) intermediate (Rai I/II, Binet B) and c) advanced (Rai III/IV, Binet C) stage disease [6]. However, these systems are of limited prognostic value in the early stage of the disease, with applies for most of the patients at diagnosis. The biologic mechanisms involved in the clinical progression from early stages of patients with chronic lymphocytic leukemia are not well known. Dysfunctional apoptosis and cell cycle are the main reasons for the clinical enigma, that CLL cannot yet be cured with conventional chemotherapy. However, the molecular pathways that are responsible for this characteristic feature of the B-CLL cells still need further definition.

We have recently demonstrated that TNF-alpha gene polymorphism did not associated with the CLL manifestation in patients with CLL [7]. The purpose of the present study was to determine whether functionally relevant polymorphism within the *INFG* gene could contribute to the pathogenesis of B-CLL.

Materials and Methods

Patients and Control

Sixty one patients F/M 25/36, aged 39–85 median 70 years with B CLL were investigated. B CLL was diagnosed according to defined clinical, morphological and immunological criteria.

Patients were treated at the Department of Haematology, Wroclaw Medical University. At the time of the study 30 patients were untreated, 22 patients had been previously given chlorambucil, whereas each cases had been treated with more intensive protocols comprising cyclophosphamide, adriamycin, vincristin and prednisolone. According to the Rai/Binet classification, there were 16, **1**, 28, 3 and 7 patients in stage 0/A, 0/I/A, I/A, II/A, and II/ B of the disease respectively. The other six patients presented with more advanced disease 3, 2, and 1 with stage III/B, III/C and IV/C respectively (Table 1). In addition 50 healthy individuals of both sexes served as a control group.

IFNG Genotyping

DNA was isolated from the whole blood taken on EDTA with the use of Qiagen DNA Isolation Kit (Qiagen GmbH, Hilden, Germany).

Biallelic polymorphism (A/T substitution) within the IFN-gamma gene (*IFNG*) was determined by PCR-SSP technique employing commercial primers (One Lambda, Inc. Canoga Park, CA, USA). In brief, for each polymorphic site one PCR reaction was carried out on DNA template with a pair of specific primers, the additional control primes, reaction mix (provided by a manufacturer), and Taq polymerase (Invitrogen, USA) in a total volume of 10 μl. Amplifications were performed in MJ Research Apparatus (Watertown, MA, USA). PCR cycling conditions were as follows: 96°C for 130 s, 63°C for 60 s, followed by nine cycles of 96°C for 10 s, 63°C for 60 s, and followed by 20 cycles of 96°C for 10 s, 59°C for 50 s, 72°C for 30 s, ending with 4°C. PCR products were analysed electrophoretically in 2% agarose gel and visualised under UV.

Table 1 Patients' characteristics. All the patients were diagnosed with CLL of B phenotype

Patients, *n*		61
Aged (range), median, yrs		(39–85), median 70
Sex, F/M		25/36
Stage of the disease (Rai/Binet)		
Early	0/A	16
	0/I/A	1
	I/A	28
	II/A	3
Intermediate	II/B	7
Advanced	III/B	3
	III/C	2
	IV/C	1

Statistical Analysis

Genotype and allele frequencies were compared between the study groups by the Fisher's exact test. Probability values <0.05 were considered statistically significant.

Results and Discussion

In this study B-CLL patients and healthy individuals presented with different frequencies of IFNG genotypes (Table 2). The patients were more frequently IFNG AA homozygotes (0.38 vs. 0.14, $p=0.004$) while less frequently were carrying the IFNG heterozygous genotype (0.40 vs. 0.68, $p=0.027$). No significant difference was observed in the distribution of the TT homozygosity in CLL patients and controls.

We also compared the distribution of IFNG alleles and genotypes in patients with respect to Rai and Binet classification. However, no significant differences were observed (individual data not shown).

Thus, distribution of IFNG (874 A/T) polymorphic features appeared to differ in CLL patients and healthy individuals of the present study and the presence of the IFNG AA homozygous genotype was found to prevail in patients as compared to controls. These results suggest that IFNG AA homozygosity could be associated with susceptibility to B-CLL in Polish patients with this disease (23/61 vs. 7/50, $p<0.005$, for patients and controls, respectively). No relationship of IFNG alleles with clinical outcome/progression of the disease was observed.

The recent studies on the role of IFN-γ in B-CLL have been mainly based on the analysis of the intracellular production of this cytokine assessed with the use of flow cytometry. These studies documented rather increased IFN-γ production by both CD4 and CD8 T cells in patients as compared to healthy individuals [8–12] The differences were found in frequencies and intracellular levels [mean fluorescent intensity (MFI)] of IFN-γ producing T cells. Additional analysis showed no difference in IFN-γ production between B-CLL patients with indolent and progressive disease [9]. However, a significant difference was observed in number of T cells spontaneously producing this cytokine in patients with progressive disease as compared to healthy donors and patients with non-progressive CLL [10].

In contrast, Gallego et al. [13] did not found any significant differences in TNF-α or IFN-γ intracellular expressions between patients and healthy individuals. They observed that TNF-α- and IFN-γ-expressing CD8 T cells were disease stage dependent, being significantly higher in late-stage patients.

As for the Polish patients with CLL, Podhorecka et al. analysed IFN-γ production capacity of T-cell subsets and B lymphocytes in B-CLL patients in comparison with healthy individuals and during disease progression [11]. As in the other studies, they detected statistically significantly higher percentage of both CD3+/CD4+/IFN-γ+ and CD3+/CD8+/IFN-γ+ cells in patients than in controls. Moreover the percentage of CD3+/CD8+/IFN-γ+ cells correlated with stage of the disease and parameters of disease progression like lymphocyte count and total tumour mass score. By contrast, the percentage of CD19+/IFN-γ+ cells in B-CLL group was lower than in control. These findings indicated that the subset of CD3+/CD8+ cells expressing IFN-γ seemed to play a special role in the disease progression as T-cell populations rather than malignant B cells appeared to be the source of IFN-gamma in B-CLL patients.

The IFN-γ production (especially by T cells) appeared to have a significant effect on the susceptibility to CLL and we found that functionally relevant polymorphism within the IFN-γ encoding gene could contribute to the risk of this malignant disease. The presence of the IFNG AA genotype was found to be associated with susceptibility to CLL.

Using a number of different assays, several groups investigated the effect of short tandem repeats and single nucleotide (+874) polymorphisms on IFN-γ production (reviewed by Warle et al. [14] and Vanderbroeck and Goris [15]). It was generally found that individuals homozygous for STR allele 2 (or having T at polymorphic position +874), in contrast to those carrying allele 3 (or +874 A), characterize with a greater IFN-γ secretion by peripheral blood mononuclear cells (PBMC) upon different mitogen stimulation [13, 14, 16].

These data suggest that individuals rather less prone to the higher IFN-γ production (low or intermediate producers of this cytokine) appear to be more susceptible to this

Table 2 Distribution of the IFNG genotypes in CLL patients and healthy individuals

IFNG alleleas and genotypes	CLL patients ($n=61$) n (%)	Controls ($n=50$) n (%)	p
AA	23 (38%)	7 (14%)	0.004
AT	24 (40%)	34 (68%)	0.027
TT	14 (23%)	9 (18%)	NS
A	47 (77%)	41 (82%)	NS
T	38 (62%)	43 (86%)	0.004

malignant disease. Therefore, it could be hypothesized that maybe not only the defect in the response to cytokine produced by T cells that regulate apoptosis, but also genetic predisposition to lower IFN-γ generation potential could contribute to the development of CLL. This preliminary results needs o be confirmed in the more extended study, including patients from the other centers.

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